α-Tomatine Extraction from Green Tomatoes

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Unripe tomatoes represent an agri-food waste resulting from industrial by-processing products of tomatoes, yielding products with a high content of bioactive compounds with potential nutraceutical properties. The food-matrix biological properties are attributed to the high steroidal glycoalkaloid (SGA) content. Among them, α-tomatine is the main SGA reported in unripe green tomatoes.

Keywords: green tomatoes ; glycoalkaloids ; α-tomatine

1. Introduction

Tomatoes (*Solanum lycopersicum* L.) are among the most cultivated and consumed vegetables worldwide. The Food and Agriculture Organization Corporate Statistical Database (FAOSTAT) reported tomato global production exceeding 180 million metric tons in the two-year period of 2020–2021 [1]. Tomatoes are consumed fresh or processed in a wide variety of products including sauces, creams, concentrates, dried fruits, and pastes [2]. As a consequence of this massive demand, tomato industries produce about 15 million tons of waste pre- and post-processing [2]. The pre-processing waste, amounting about 1–6% of the total tomato production, includes damaged tomatoes and green tomatoes [3]. During red tomato harvesting, green tomatoes are separated by a color-sorting machine and discarded. The main post-processing waste is tomato pomace, a mixture of seeds, peels, fibrous parts, and a small part of the pulp [4]. This waste represents up to 2 million tons of organic material and about 5% of processed tomato weight [5]. Tomato agricultural by-products represent a disposal cost for industries and affect the environment. Therefore, governments promote the recovery of agricultural wastes, granting campaigns aimed at by-product recycling [6][7].

Green tomato waste is mainly recycled to prepare cocktail sauces, snacks, and other food products [8]. Green tomato juice can be also used to replace sugar-rich juice for the preparation of beverages or for animal feeding [9]. However, considering the nutraceutical properties of its secondary metabolites, tomato waste is being used for the isolation of natural compounds to include in supplements, additives, and pharmaceuticals. Red tomato waste has been extensively used for the extraction of lycopene [10]. Differently, green tomatoes are just starting to be considered for pharmaceutical or nutraceutical formulation, despite unripe and ripe tomatoes both displaying interesting nutraceutical components [11]. The main composition differences between the green and red maturation stages are related to secondary metabolite classes, such as steroidal glycoalkaloids (SGAs), polyphenols, carotenoids, ascorbic acid, and chlorophylls. The polyphenolic content is highly variable between green and red tomatoes. High polyphenolic contents have been reported in green tomatoes, particularly for chlorogenic acid and rutin [12]. The content of the two components decreases as the fruit ripens, but increases in ripe red tomatoes. In this stage, the main polyphenols are naringenin and homovanillic acid [12]. Carotenoids represent a class of distinctive tomato secondary metabolites that differ qualitatively and quantitatively according to the degree of fruit ripeness. The most abundant carotenoid in immature tomatoes is lutein (about 45% of the total carotenoid content), but violaxanthin, neoxanthin, and beta-carotene are also representative [13]. In ripe red tomatoes, lycopene is the most distinctive, accounts for between 59 and 91% of total carotenoid content, and gives the typical red color; beta-carotene and lutein are also relevant [14]. The high lycopene content of ripe red tomatoes, which is completely absent in unripe green tomatoes, justifies the use of waste products from ripe tomatoes as a source for the extraction of carotenoids [13]. On the contrary, green tomatoes are rich in essential nutrients such as vitamins (e.g., C, K, and B-complex vitamins), minerals (e.g., potassium, magnesium), and dietary fibers, all contributing to a balanced diet. Ascorbic acid, known as vitamin C, is a powerful antioxidant with several benefits to human health. The content of vitamin C in tomato fruits is dependent from the degree of ripeness [8]. Green tomatoes display a vitamin C content of about 25 mg/100 g of fresh fruit. This content increases as the fruit ripens, but slightly decreases in the ripe red tomato stage, reaching a content of about 10–20 mg per 100 g of fresh fruit [15]. However, data in the literature do not agree on the link between vitamin C content and the degree of ripeness [11]. Green tomatoes also contain phytochemicals like β-carotene, as well as chlorophyll, with antioxidant and anti-inflammatory properties. Therefore, they reduce the risk of dysmetabolism

and cardiovascular diseases as diet supplements [16][17][18]. Chlorophylls are secondary metabolites that give the typical green color to unripe fruits. Their content decreases dramatically as the fruit ripens [8].

2. Unripe Green Tomatoes as a Source of Glycoalkaloids

SGAs are a class of secondary metabolites produced by some plants in the Solanaceae family, such as tomatoes, potatoes, and eggplants. They are distributed in different botanical parts, which include the leaves, flowers, stems, roots, and unripe fruits [19]. Tomatine was firstly isolated by Fontaine et al., in tomato leaves and represents the main SGA isolated from the whole tomato plant [20]. Tomatine was originally isolated as a mixture of two SGAs, α-tomatine and dehydrotomatine, which differ based on a double bond in the aglycon moiety across positions 5 and 6. However, the chemical composition of tomatine commercial standards were characterized as a mixture of 90% α-tomatine and 10% dehydrotomatine in 1994 [21]. Therefore, all the studies conducted up to that year on tomatine actually referred to a mixture of the two compounds, presumably with the ratio 9/1. α-tomatine ($C_{50}H_{83}NO_{21}$; molecular weight: 1034.2) is characterized by a nitrogen-containing spirostanic aglycone, called tomatidine ($C_{27}H_{45}NO_2$; molecular weight: 415.7), attached to a tetrasaccharide unit in position 3β (*S* configuration). It is referred to as lycotetraose, which consists of two D-glucose and single D-galactose and D-xylose units [22]. The chemical structure is reported in **Figure 1**. α-tomatine belongs to the class of phytoanticipins, which are natural antimicrobial compounds that protect the plant against potential pathogens as fungi [23]. Although α-tomatine represents the most abundant tomato SGA, several secondary metabolites have been identified, both as biosynthetic precursors and as products of α-tomatine metabolism in the plant. The chemical diversity of α-tomatine derivatives is derived from some chemical modifications, such as isomerization, hydroxylation, acetylation, and glycosylation [24]. α-tomatine is present in all tomato parts, and the fruit concentration depends on the stage of maturity. Indeed, this compound is accumulated in unripe and green tomatoes, whereas during fruit maturation, it drastically decreases [25]. During the transition from green to red fruit, it is metabolized to esculeoside A, which represents the main mature tomato SGA [24].

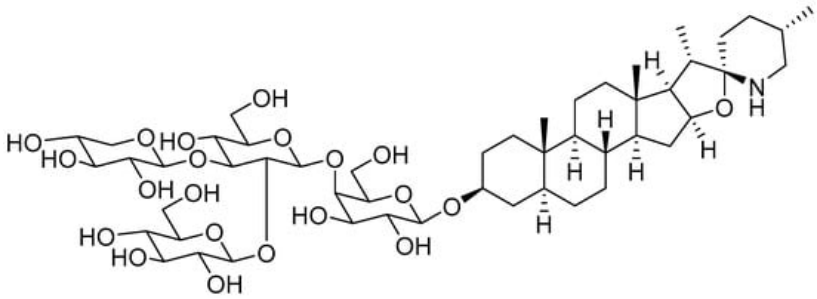

Figure 1. Chemical structure of α-tomatine. Structure was drawn using the chemistry software ChemDraw Professional 15.0.

3. α-Tomatine Extraction from Green Tomatoes

α-tomatine extraction represents a key process for the correct titration of unripe tomato extracts. Tomato SGAs are usually extracted by grinding the samples with a mortar and pestle, or blending and then extracting analytes with polar solvent systems [26]. The protocols for unripe tomato analysis involve adding extraction mixtures to a specified amount of fresh tomatoes or lyophilized powdered tomatoes and stirring the mixture for a certain period at a controlled temperature. The most common extraction solvents are acidic aqueous or organic solutions (e.g., methanol, acetonitrile–methanol, methanol/chloroform, or tetrahydrofuran). SGAs are basic compounds due to the nitrogen atom in the spirostanic ring. Indeed, α-tomatine solubility in water depends on the pH of the solution (6 mM at pH = 5; 1 mM at pH = 6; 0.04 mM at pH = 7; and 0.03 mM at pH = 8) [27]. Acetic acid improves α-tomatine extraction by protonating the nitrogen atom and increasing the water solubility. However, acetic acid is mainly used in dried samples, while organic solvents (e.g., methanol or chloroform) are preferable with fresh tomatoes. Indeed, fresh samples contain a water content that is sufficient to solubilize the alkaloids during the extraction [28]. After the extraction, the solution can be (1) centrifuged to remove the precipitated components [21][29][30][31][32][33][34][35][36][37][38][39]; (2) concentrated under vacuum and acidified with a solution of hydrochloric acid (0.2 N) [40][41][42]; (3) or subjected to both procedures [43][44]. The samples obtained can be directly analysed [29][32][36][38] or subjected to partial purification procedures. The most common approaches to partial purification include (1) the clean-up of the samples with solid-phase extraction (SPE) [21][30][31][33][34][35][42][45][46][47][48]; (2) the precipitation and centrifugation of SGAs in basic conditions through the addition of an ammonium solution [14][40][41][43][44][49]; (3) or liquid–liquid extraction (LLE) with organic solvents [21]. The purification approach with ammonium hydroxide allows SGAs to be selectively purified. However, the precipitation is not quantitative and is characterized by a low recovery rate [28]. Instead, the SPE purification approach allows for greater reproducibility, but requires the validation of

the recovery parameters for a correct quantification [42]. Various SPE cartridges are commercially available and are used in SGA analysis. The most common sorbent type is the octadecyl phase (C_{18}), but other SPE sorbents include a sulfonic acid cation exchanger (SCX), a macro porous copolymer (Oasis HLB), a polar cyanopropyl (CN), aminopropyl bonded silica (NH_2), combined packings (CN/SiOH; NH_2/C_{18}), and mixed sorbent phases as a combination of octyl and SCX (Certify) [50][51]. The SPE clean-up procedures include a first step of absorption of SGAs to the sorbent, a second step of washing the impurities off the phase, and finally, the elution of SGAs with an organic solvent, generally methanol. Impurity washing can be performed with water to remove fibers and sugars, or with aqueous organic and ammonium mixtures to elute acidic interferents as polyphenols [21][34]. The C_{18} SPE requires the loading of only aqueous extracts to allow the absorption of SGAs at the hydrophobic phase. Therefore, the C_{18} SPE is frequently suitable for acidic aqueous extracts that require a clean-up or the elimination of possible interferents in sample analysis [30][42][48]. The extracts obtained with organic solvents require the evaporation of the organic solvent and then its solubilization in water before loading on the cartridge. To ensure SGAs' solubility and the loading of the sorbent cartridge, heptanesulfonic acid can be added as an ion-pairing reagent, improving the linkage with the C_{18} SPE phase [52]. α-tomatine recoveries with different types of SPE are greater than those of other SGAs (e.g., α-solanine, α-chaconine) and are close to 100%. However, the use of the SCX sorbent phase ensures greater selectivity for SGA purification, allowing a reduced matrix effect during the analysis. The sulfonic acid group is strongly acidic and interacts with basic species, as the nitrogen group of SGAs, improving the efficiency of the purification [33][42]. The SCX SPE requires different conditions for the interferents' clean-up and the elution of SGA fractions compared to C_{18} SPE purification. The clean-up is carried out using aqueous organic mixtures of methanol, generally at 5–10%, which remove non-basic interferents such as polyphenols. Instead, SGA elution is performed with basic ammonia mixtures in organic solvents (e.g., 2.5–5% ammonium in methanol) [53][54].

Another approach for sample clean-up includes the liquid–liquid extraction (LLE) of the aqueous ammonia solution with a water-saturated 1-butanol solution to recover the 1-butanol layer enriched by the SGA fraction. 1-butanol LLE extraction can be used after a C_{18} SPE clean-up to obtain a robust purification of the samples from matrix interferents [34]. Ultrasound-assisted extraction (UAE) is commonly associated with the other extraction steps to improve the extraction yield. UAE is a flexible, low cost, simple, and scalable non-conventional technique. It is based on the cavitation principle, which allows cell wall disruption with the extraction of bioactive compounds. The extraction procedure can also be conducted in high throughput in order to reduce the analysis times and maximize the extraction yield. A validated method suitable for α-tomatine and tomatidine extraction has been described, requiring an approximate preparation time for each sample of 1.25 min, with a α-tomatine extraction recovery close to 100% and without clean-up procedures [26].

An essential point in the analytical validation of an extraction is the recovery study of the active ingredient from the matrix. Although several papers report methods for α-tomatine extraction, few authors have adequately supported them with recovery studies. However, it seems that using acidified solvents guarantees a very high recovery and adequate analytical accuracy [29][33][52].

4. α-Tomatine Analysis Methods

Over the years, several techniques have been applied for α-tomatine identification and quantification. High-performance liquid chromatography (HPLC) is the analytical approach that has been the most widely employed. One of the first detectors coupled with HPLC that was used for α-tomatine analysis was gas chromatography [55]. Due to the polarity of this compound, it could not be analysed directly in an intact form, but required hydrolysis reactions of the sugar component and functionalization to increase the volatility [55]. The analysis can be carried out either on lycotetraose sugars or on aglycones. For example, an identification protocol through SGA hydrolysis was described based on the reduction of monosaccharides to alditols, which are subsequently acetylated [35].

The most commonly used approach for the analysis of α-tomatine is liquid chromatography combined with an electron spray ionization mass spectrometer (LC/ESI-MS), a quadrupole time-of-flight mass spectrometer (Q-TOF MS), and Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) [24][31][46][56][57]. The soft ionization of these techniques allows for the analysis of highly polar, high-molecular-weight, and thermally unstable compounds in their intact form [11]. Furthermore, SGA nitrogen atoms make the analysis extremely sensitive in positive acquisition mode, achieving a sensitivity with an α-tomatine LOQ value of 1.1 femtomoles [26]. Qualitative experiments (e.g., full MS, MS/MS, DDA) are the most widely used approach for the identification of α-tomatine and other SGAs from food matrices [31][37] and biological samples [58][59][60].

Although gas chromatography (GC) and mass spectrometry (MS) are widely used approaches for α-tomatine analysis, they are not often available in laboratories. Therefore, some analytical methods have been developed with reverse-phase high-pressure liquid chromatography (RP-HPLC) using other detectors, such as pulsed amperometry (PAD), ultraviolet

(UV), diode arrays (DADs), evaporative light scattering (ELS), and refractive index detectors (RIDs). The pulsed amperometric detection (PAD) represents one of the first techniques used for α-tomatine analysis. This detector was used for the first quantitative analysis of α-tomatine in tomatoes [21], the different parts of the plant [35], and processed tomato products [34]. Although PADs are a more sensitive analytical technique than other detectors (e.g., UV and DADs), HPLC analysis can generate a non-linear response due to the overloaded detector cell at high concentrations [35]. The ultraviolet (UV) and diode array (DAD) detectors are the most widely used techniques due to their easy use and low cost of analysis [33][40][42][43][45][48][54][61]. However, α-tomatine is detected around 200 nm due to the lack of UV-visible functional groups, a wavelength with low specificity. Therefore, HPLC analysis requires the use of clean-up techniques (e.g., SPE) to reduce the number of possible interferents and is characterized by low sensitivity.

Although RP-HPLC methods represent the most common approach for the analysis of α-tomatine and other SGAs, several alternative methods have been developed over the years for the purification, identification, and quantification of these molecules. Thin-layer chromatography (TLC) is a technique of direct-phase chromatography that separates SGAs based on the different polarities of sugar moieties. It is used to estimate the number of molecules present in a mixture and to monitor fractions during chromatographic separation. Therefore, this technique exhibits only a qualitative value and cannot be used for quantitative purposes. However, examples of preparative TLC or high-pressure thin-layer chromatography (HPTLC) for the isolation of α-tomatine from plant matrices are reported in the literature [62][63]. Another approach used for SGA separation was capillary electrophoresis (CE), a low-cost and -speed technique which separates compounds based on their ion mobilities. CE has often been used for the separation of ionizable basic compounds such as alkaloids [64].

5. Nutraceutical Potential of Green Tomatoes

5.1. Antiviral, Antifungal, and Antibiotic Activity

SGAs are produced by plants as a defense against bacteria, fungi, viruses, and insects [65]. It is thus not surprising that the healthy properties of green tomatoes' alkaloids are a consequence of the antibiotic power of these secondary metabolites. Leaves and immature green fruit extracts of Californian *Solanum lycopersicum* L. display antimicrobial activity against several bacteria (*Salmonella enterica*, *Staphylococcus aureus,* and *Escherichia coli* K12) [66]. Interestingly, the extract does not affect the growth of the beneficial bacteria *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, and *Lactobacillus reuteri*, which are part of the human gut microbiota [66]. A-tomatine affects the membrane permeability of many crop-infesting fungi by sequestering ergosterols, one of the main components of fungal membranes [67]. Ergosterol sequestration disrupts the membrane bilayer, ultimately causing the leakage of cell components, osmotic stress, and cell death [27][68]. Among SGAs, α-tomatine has the highest bactericidal activity against bacteria and fungi [67]. α-tomatine, isolated from young leaves of *Lycopersicon Pimpinellifolium*, showed activity against the pathogen *Fusarium caereleum* (IC_{50} = 460 µM). Further, α-tomatine included in bacterial Petri dishes completely inhibits the growth of fungal species, such as *Candida albicans* (α-tomatine-enriched extracts of green tomatoes, leaves, and stems) [66]; *Fusarium oxisporum* (IC_{50} = 40 µM); and *Cladosporium fulvum,* as well as the spore germination *of Paecilomyces Fumosoreus* (IC_{50} = 500 µM), and partially reduced of 45% the spore germination of *Beauveria brassiana* (IC_{50} = 1 mM) [69][70]. In the fungal pathogen *Fusarium*, the damage caused by this compound increases reactive oxygen species (ROS) production and leads to fungal programmed cell death [71].

5.2. Anti-Inflammatory Effects

Several articles have reported the anti-inflammatory effects of pure SGAs and green tomatoes' extracts. Extracts obtained from the locular gel and serum of *Solanum lycopersicum* L. var. "Camone" (respectively, containing 61.7 ± 0.9 mg of α-tomatine/kg of FW of locular gel and 12.5 ± 0.5 mg of α-tomatine/kg of FW of serum) significantly reduce inflammation in humans, decreasing the blood inflammatory cytokine count, systolic pressure, heart rate, and aorta thickness [36]. The supplementation of 1–2% dietary tomato powder containing α-tomatine ameliorates hemato-immunological and antioxidant clinical parameters in rabbits [72].

Pure α-tomatine inhibits the production of the proinflammatory cytokines IL-1β, IL-6, and TNF-α in LPS-stimulated macrophages by preventing IκB degradation and ERK phosphorylation [73]. In agreement with these reports, α-tomatine has been shown to inhibit the expression of Cox-2 and iNOS and decrease the production of prostaglandin E2 (PGE2) in murine LPS-stimulated macrophages. Furthermore, α-tomatine exerts a powerful antihistaminic effect [74].

5.3. Anti-Aging Effects

Green tomatoes and SGAs have shown promising anti-aging effects in many tissues, including the bones, brain, and muscles. A diet supplementation with a green tomato extract from "*Korean chal tomato*" (containing tomatidine in the amount of 1.06 ± 0.11 mg of tomatidine/100 g of dry weight) improved bone mineral density and overall bone quality in ovariectomizes rats, a model of postmenopausal osteoporosis [54]. The aglycone tomatidine inhibits osteoclastogenesis and reduces estrogenic deficiency-induced bone mass loss [75] through a mechanism that has not been fully elucidated, but that probably involves the modulation of the p53 and MAPK signaling pathways [76].

5.4. Anti-Tumoral Effects

Red tomato extracts from the fruits of variants of *Solanum lycopersicum* L. (var. *Sancheri premium*, *Yoyo*, *Chobok Power,* and *Rokusanmaru*) have only subtle growth inhibitory effects in several in vitro cell culture models (breast cancer (MCF-7), colon cancer (HT-29), gastric cancer (AGS), hepatocarcinoma (HepG2), and liver cancer (Chag)). However, the corresponding extracts from unripe green fruit (α-tomatine content ranging from 5.75 ± 0.29 mg of α-tomatine/100 g of FW to 31.40 ± 1.97 mg of α-tomatine/100 g of FW) inhibit the growth of several human cancer lines, such as the MCF-7, HT-29, AGS, HepG2, and Chag lines [44][77].

5.5. Pharmacokinetics and Toxicological Aspects of Glycoalkaloids and Green Tomato Extracts

The correlation between in vitro pharmacological activities and possible beneficial effects on human health is strictly correlated to the study of α-tomatine pharmacokinetics (e.g., absorption, distribution, biotransformation, and excretion). Although α-tomatine toxicology and the in vivo fate of other SGAs (e.g., α-solanine, α-chaconine) have been extensively studied [78], there are few data about the pharmacokinetics of tomato SGAs. For many years, α-tomatine was considered a molecule with low bioavailability. It is stable at 37 °C under acidic conditions that mimic the pH of the stomach. Furthermore, α-tomatine and cholesterol form insoluble complexes that are eliminated through feces [65].

6. Conclusions

Waste products of the tomato industry represent a rich, natural source of α-tomatine, and the recycling of this waste represents an appealing research field to develop innovative nutraceutical products. It is thus not surprising that over-the-counter products containing green tomato extracts are starting to become popular. Among their secondary metabolites, α-tomatine exhibits significant biological activities on human health. The health-beneficial properties of pure tomato compounds (e.g., α-tomatine and tomatidine) and *Solanum lycopersicum* L. extracts in several diseases have been discussed. Besides its antioxidant power, α-tomatine-containing extracts show interesting antimicrobial, anti-inflammatory, anti-aging, and anti-tumoral activities. In vitro, the cellular and molecular mechanisms involved in green tomato pharmacological activities have been identified and proven to involve the modulation of several metabolic patterns.

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