Development of a cascade production system finalized to the extraction of all-tomatine-rich fraction using the tomato cannery waste as feedstock

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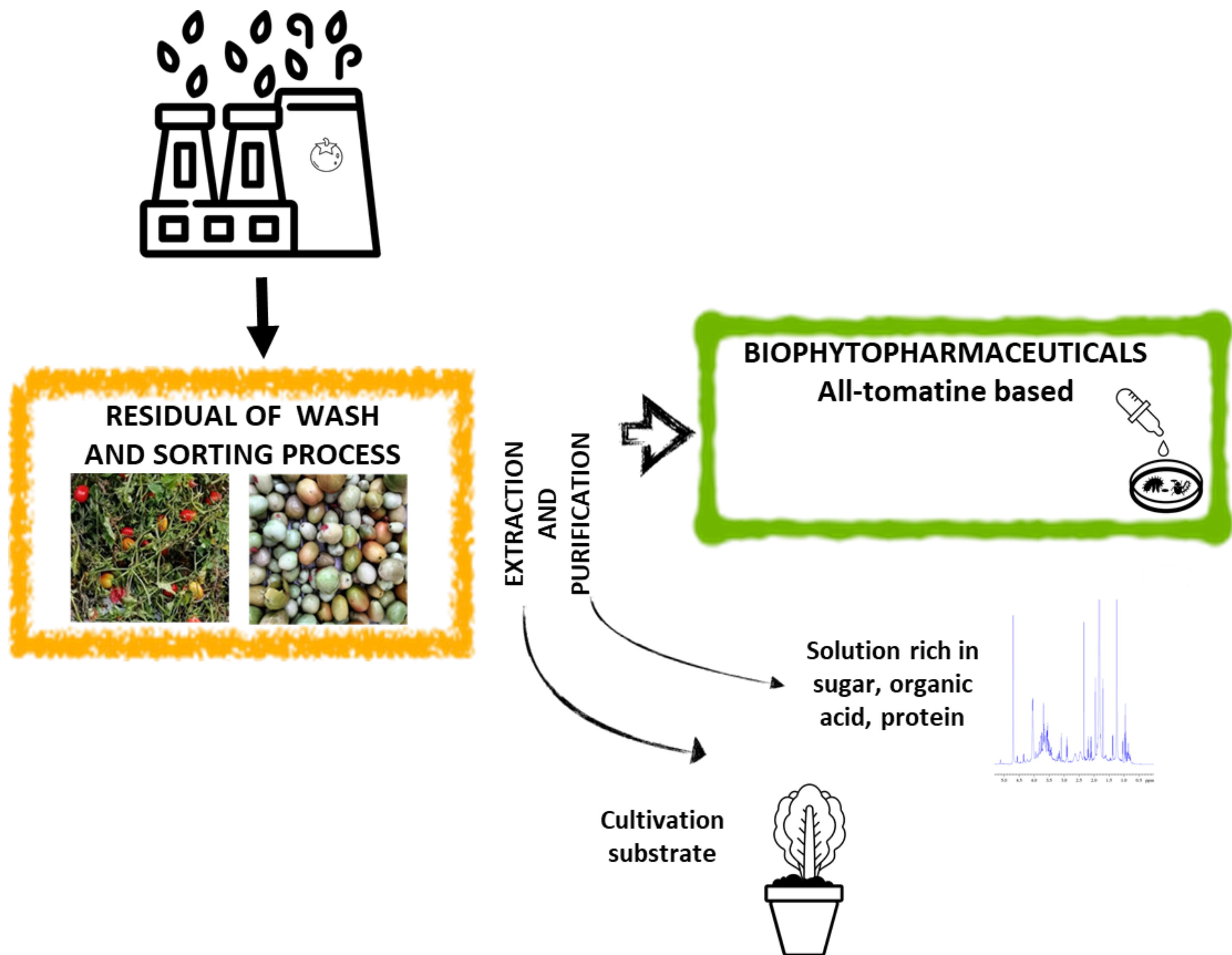
GRAPHICAL ABSTRACT



Abstract

Tomato plants produce, among other defensive molecules, glycoalkaloides (i.e. all-tomatine). All-tomatine, found in highest concentrations in green fruits, leaves and stems, has demonstrated a wide variety of biological activities useful in fields such as agronomy and biopesticides. Despite the interest in its potential use, extraction methods for all-tomatine are few and mostly developed with an analytical scope. In analogy with other active principles (pyrethroids, nicotinoids), the all-tomatine can represent an alternative to the use of synthetic pesticides currently discouraged for their environmental persistence and progressive reduction of efficacy against target organisms.

The aim of this work was to attempt the development of a process to extract all-tomatine based on the re-use of tomato waste industry residues as feedstock and to apply a cascade production approach. To do so, the lab-scale methods to extract all-tomatine were employed as a starting point and different tomato portions (green fruits, leaves, and stems) as feedstock. The best process in terms of feasibility and recovery was identified by mixing the extraction (acetic acid 5% v/v) and purification (ammonia 25% v/v precipitation) steps of different methods. The process was successively tested using the residues of the full-scale tomato cannery plant waste composed by stems+leaves (SL), and green fruits (GT) currently not valorized. The best recovery was obtained by the SL (yield of the extract: 10.8 mg g^{-1} dry matter (DM) of the starting biomass; all-tomatine pureness: 864 mg g^{-1} DM extract). The use of the acetic acid as extraction agent gave a solvent-free by-product (68% DM starting biomass), reusable as cultivation substrate. At the purification phase, the dialysis-treatment of the wastewater, recovered a solution rich in sugars and organic acid to get a full starting biomass recovery of 85.6% DM starting biomass.

Keywords: tomatine, cascade approach, tomato waste, ammonia precipitation, NMR, biopesticide.

Highlights:

- Tomato plants produce all-tomatine as a defense metabolite.
- All-tomatine is interesting as a biopesticide.
- The residues of the tomato cannery industry were tested as feedstock
- All-tomatine was extracted with acetic acid and precipitated with ammonia
- The process applied a cascade approach with a high biomass recovery.

Abbreviation: SGAs (steroidal glycoalkaloids); SPE (solid phase extraction); SL (stems leaves); GT (green tomato fruit); BP (biopesticide), E extract (precipitate rich in all-tomatine), HPLC (high pressure liquid chromatography); SCX (Strong Cation Exchange resin), NMR (Nuclear magnetic resonance spectroscopy).

1. Introduction

The steroidal glycoalkaloids (SGAs) class of compounds include secondary metabolites produced by plants that have a protective role against plant pests and diseases such as insects, bacteria, and fungi (Friedman, 2002); they contain a nitrogen group in a steroid skeleton, which is the most characterizing portion, coupled in some form to one or more monosaccharides.

The SGAs produced by tomato plants (*Solanum lycopersicum*), are named tomatine (all-tomatine) and include approximately 100 different molecules. The two most well-known of these tomatines are tomatidine and tomatidinedol, two very similar aglycate forms, the latter has a double bond between C5-C6 (Cataldi et al., 2005), and the correspondent glycosylate forms – named α-tomatine and tomatidine, respectively – in which saccharides portion is made by four carbohydrate (Gal:Glu:Xyl as 1:2:1) (Friedman, 2002).

The quali-quantitative composition of all-tomatine differs among different organs and changes at different developmental stages of tomato plants, moreover it is influenced by seasonality and other external conditions. The highest total concentration of SGAs was measured in flowers (3000-5000 mg kg^{-1} wet weight –w w) (Kozukue et al., 2004) but, in terms of dry matter (DM), the highest value is found in immature green tomatoes (743 mg kg^{-1} DM) followed by stems, calyxes, and leaves (Friedman, 2002). Tomato varieties are also an important variable as, for example, very high α-tomatine and tomatidine concentrations were found in the Andean cherry tomato and Mini tomatoes, with a concentration of 6300 mg kg^{-1} DM and 1498 mg kg^{-1} wet weight (ww) respectively (Rick al., 1994; Kozukue et al., 2004).

The interest in all-tomatine is due to their bioactivity i.e. ability to interact with one or more component(s) of the living tissue to give a positive effect on human health or to act as biocide against, fungi, bacteria and insect. In particular, α-tomatine showed anti-inflammatory, anti-

cholesterol and anti-cancer properties (Friedman, 2006; Friedman et al., 2009; Milner et al., 2011; Friedman, 2013; Serratì et al., 2020); as well as biocidal effect against pathogenic bacteria (*Listeria monocytogenes Staphylococcus aureus, Salmonella enterica, Escherichia coli*), fungi and insects (Pingulkar & Kiran, 2001; Friedman, 2002; Friedman, 2013; Bailly, 2021; Zhao et al., 2021).

More recently, some bioactivities (i.e. anti-ageing, anti-tumour, anti-inflammatory, anti SARS-CoV-2 infections, phytotoxicity, and fungicidal activities) have been discovered for tomatidine as well (Woods et al., 2004; Simons et al., 2006; Chiu & Lin, 2008; Guay et al., 2018; Yu et al., 2020; Troost et al., 2020; Huang et al., 2021; Zrieq et al., 2021).

Still, most of these results were obtained using analytical standards and extremely purified forms of these molecules, while very few studies tested the effects of all-tomatine extracted from natural sources using leaves, stems, and green fruit as feedstock, which would result in extracts composed by mixtures of all-tomatine plus other molecules.

In those last cases the tomatine extracts were obtained by three principal steps: sample preparation (drying or freeze-drying plus milling), extraction and purification (Taveira et al., 2012; Tamasi et al., 2019). The extraction is usually performed with methanol or ethanol (with mechanical stirring and/or ultrasound) in some cases in conjunction with acids (Tamasi et al., 2019). As an alternative, Taveira et al. (2012) tested the use of diluted acetic acid. Likewise, two different purification processes were found: the most common (75% of the consulted methods) was through solid phase extraction (SPE), the alternative being the use of ammonia to precipitate all-tomatine (Kuronen et al., 1999; Kozukue et al., 2004; Friedman et al., 2009). These previously described methods were designed for research application as, to the best of our knowledge, the actual extraction of all-tomatine from biomass is limited to scientific purposes, although some patents have been registered for their production and successive employment as biopesticides (BP) in agriculture

(CN101032246A, Year: 2007; JP2020203856A, Year: 2010; CN112120225A, Year: 2020) (Google Patents, 2022).

In the latest years, the use of synthetic pesticides has been progressively discouraged by policies (D.L. n. 150 14/08/2022, DM 22/01/2014-PAN) in response to increasing evidence regarding their persistence in the environment, adverse effects on human health, and progressive reduction of efficacy against target organisms. This contributes to a scenario in which the research of new, effective, and low environmental impact active substances is of major importance. Some well-known insecticidal molecules (pyrethroids, nicotinoids, etc...) derive from plant biomass and are currently in use, however, the use and development of alternative BP require continuous research process to contrast the progressive reduction of efficacy against pathogens and pests.

The development of a new biopesticide production and use are, instead, based on the process's sustainability (Fenibo et al., 2021). The use of discharged biomasses is interesting in terms of available amount to improve the process feasibility and reduce the cost. Regarding tomatine, a great opportunity comes from the tomato canning sector, a very relevant food industry worldwide and of particular interest to Italy, being the largest producer in Europe (51% of total production in EU) with 5595 million tons of processed products in 2020 (https://agriculture.ec.europa.eu/data-and-analysis/markets/production-data/production-sector_en), and the third one in the world (13% of total production worldwide) after United States and China with an economic relevance of 3.5 billion euros (https://news.italianfood.net/2020/12/09/canned-tomato-italy-leads-the-european-ranking/).

The production of all processed tomato-based goods has a first selection step during which suitable tomato fruits are kept, whereas the unsuitable fruits and plant portions are discarded. The output of this selection gathers fractions rich in all-tomatine (leaves, stems, and green fruits) as waste

fractions (2-3% ww of the starting tomatoes). Very few information is reported about the re-use of these fractions, currently scarcely valorized and destined to the composting, feed, or biogas sectors whilst most attention was focused on the tomato pomace (peel+seed), generated during tomato juice extraction. The tomato pomace (2-3% ww of the starting tomatoes) is rich in lycopene, the most powerful antioxidant carotenoid widely required by the cosmetic, food, and pharmaceutical industries (Zuorro et al., 2014). Moreover, other phytochemicals are present such as unsaturated fatty acids, phytosterols, and tocopherols. In order to meet the growing energy request, the tomato pomace is used as feedstock for the anaerobic digestion to produce methane (Scaglia et al., 2020) but the relevant oil content (Squillace et al., 2020) suggested its possible employment as biodiesel source similarly as what reported for other vegetal wastes from food or not food crops (Chuah et al., 2016; Chuah et al., 2017; Casa et al., 2021; Rozina et al., 2022; Abbasi et al., 2023; Arshad et al., 2023; Munir et al., 2023).

A core aspect of sustainability of the new process development is the possibility to obtain more products from the same resource and reducing waste, thanks to the valorization of the exhausted fraction of the production steps in large part linked to the possibility of obtaining solvent-free residual fractions (Müller et al., 2020). Considering the lab-scale methods as starting point, the main objective of this study was to develop a process to extract an all-tomatine rich-fraction to be employed as biopesticide. To improve the feasibility of the process the tomato cannery waste residues were tested as feedstock and the residues of the steps of the production evaluate as additional products (cascade approach).

2. Material and Methods

2.1. Set up of the analytical method for all-tomatine analytical characterization

The analytical detection of samples was performed using the methods reported by Taveira et al. (2012) with some modification. The analyses were carried out using an HPLC system equipped with a UV detector (Agilent) supplied with a Luna Omega Polar C18 column (150 x 3.0 mm, 3 µm particle size, Phenomenex, Torrance, USA). The mobile phase used was acetonitrile and trimethylammonium phosphate (TEAP, 25 mM) at pH = 3, the chromatograms were recorded at 205 nm, with an elution flow of 0.4 mL min^{-1}, and the column temperature was at 30 °C. The elution solvent was a mix of acetonitrile and TEAP as follow: in the ranges 0-12 min, 12-17 and 17-20 the acetonitrile increased from 20% to 45%, from 45% to 55% and from 55% to 57% respectively (Taveira et al., 2012). The identification of the most important glycosylate and aglycate all-tomatine (i.e. α-tomatine and tomatidine, respectively) was done using commercial standards (DBA, Milan, Italy) of α-tomatine (purity> 99.38%) and tomatidine (purity ≥ 98%).

The quantitation was based on linear regression peak area vs. concentration of both standards. To do so, 1 mg of pure standard was suspended in 1 mL of methanol successively diluted in the range of 0.05-0.5 mg mL^{-1}. All samples were solubilized by sonication for 1 h and successively filtered by nylon filter (pore diameter=0.22 µm) before the HPLC analysis. Hydrolysis experiment on α-tomatine standard was carried out as described by Kuronen et al. (1999) to confirm the picks attribution of the α-tomatine and tomatidine standards (paragraph S1).

2.2. Vegetable Materials

Tomato leaves, green fruits and stems were collected from local market waste and employed to set up the all-tomatine extraction procedure.

Moreover, discarded fraction from tomato cannery industries has been collected during the seasonal activity of a full-scale plant (Ravarino, Modena, Emilia Romagna Region, Italy) at the

beginning of August (time 1) and at the end of September 2021 (time 2). The biomasses employed come from the primary sorting activities that, based on an optical and manual separation system, reject three different residues: made by mix of the crushed red tomatoes, green tomatoes (GT), stems and leaves (SL) present as a mixture in different proportions. The GT and SL, i.e. two fractions richer in all-tomatine, were sampled (20 kg ww) at time 1 and 2 (named LS1, GT1, LS2 and GT2 respectively) as feedstock for the all-tomatine extraction. All biomasses were dried at 60 °C under vacuum until constant weight was reached and were ground in a grinder to a 500 µm particle size before the extraction and the chemical characterization.

2.3 All-tomatine extraction methods

2.3.1 Application of all-tomatine extraction method based on acetic acid extraction and successive SPE purification

The samples of green fruits, leaves and stems (160 mg dry matter DM) were suspended in 2 mL acetic acid (5%) and undertaken to the sonication (time = 30 min), then to the mechanical agitation (time = 120 min at 300 rpm), and again the sonication (time = 30 min) (Taveira et al., 2012). The samples were centrifuged at 10,000 rpm for 15 min and the supernatants were collected and successively subjected/or not, to purification step by solid phase extraction (SPE) (SOPLE-SELECT SCX, 1000 mg 20 mL^{-1}, Sigma Aldrich, Milan, Italy). The SPE columns were preconditioned by 10 mL methanol + 10 mL 5% acetic acid, washed by 20 mL 5% methanol. Then 2 mL of sample were added to the SPE column and afterward 20 mL elution (2.5% ammonia in methanol) were added. Both the purified/non-purified extracts were then evaporated to dryness in a vacuum oven at 40 °C. The residues were later dissolved in 1 mL of methanol and treated as reported in section 2.1 prior to the injection to HPLC to detect the quali-quantitative composition of all-tomatine.

2.3.2 Application of all-tomatine extraction method based on methanol+acetic acid extraction and successive precipitation with ammonia

The same samples tested in section 2.3.1 (0.5 g DM) were suspended in 5 mL of methanol:acetic acid (2% v/v) solution for 2 hours under stirring and successively centrifuged at 8000 rpm for 15 min (Friedman et al., 2009). The supernatants were collected whilst the precipitates were washed with HCl 0.2 N (4 mL) and again centrifuged at 8000 rpm for 15 min. The two supernatants were successively mixed and concentrated to 1 mL in a vacuum oven at 40 °C. At this point, 1 mL of ammonia (25% w/w) was added to the samples to enhance the all-tomatine precipitation. The reaction was carried out in water bath at 65 °C for 50 minutes then the samples were chilled overnight at 4 °C and successively centrifuged at 8000 rpm/15 min. The precipitates rich in all-tomatine (named extract, E) were separated and washed with an ammonia solution (2% v/v) twice. The extracts were dried at 40 °C under vacuum and weighted to perform the mass balance of the process. The quali-quantitative all-tomatine characterization of E was done by HPLC after resuspension in methanol.

2.4 Proposal of a method for the extraction of all-tomatine

The all-tomatine yield and pureness degree (i.e. weight of all-tomatine / weight of extract) obtained during the previous activities (2.3) were analyzed to define a new method. The extraction procedure with acetic acid (Taveira et al., 2012) was carried out on 2 g DM of LS1, LS2, GT1 and GT2. The precipitate of the extraction (named exhausted, Exh) was washed with water and dried in a vacuum oven at 40 °C for the successive characterization whilst the supernatant was treated with ammonia to precipitate the all-tomatine as proposed by Friedman et al. (2009). At the end of precipitation,

the precipitate (E) was washed with distilled water until neutral pH was reached, whilst the supernatant rich in ammonia and the waters of the E washing step were subjected to the dialysis (Dialysis tubing cellulose membrane, pore size 0.45 µm, Sigma Aldrich, Milan, Italy). The dialysis was carried out until the water washing reached a constant pH, and the no-dialyzed fraction (named NDF) was dried at 40 °C under vacuum and weighted to obtain the overall mass balance.

2.5. Chemical characterization of the biomasses

Both raw and exhausted LS and GT samples were characterized for the fiber content using the Van Soest method (Abbasi-Parizad et al., 2020). The NDF were characterized for the total nitrogen using an elementary analyzer (Elementar Rapid max N exceed, Elementar Italia s.r.l., Lomazzo, Italy), based on the analytical method of combustion "Dumas" and equipped with a thermal conductivity detector (TCD, Elementar Italia s.r.l., Lomazzo, Italy), N-ammonium and N-NO_3 content by Nanocolor kit (Macherey-Nagel GmbH & CO. KG. Duren. Germany). Moreover, the organic carbon content was determined by Springer and Klee method (Vitti et al., 2016).

2.6. NMR characterization of the extract rich in all-tomatine

The NMR spectra of extract richer in all-tomatine (from LS1) were recorded at room temperature on a Bruker AVANCE NEO 500 MHz spectrometer equipped with a 5 mm TCI cryoprobe. The ^{1}H NMR spectra of extracts dissolved in methanol were recorded using a classical *zg* sequence (Bruker standard pulses sequence). For all the ^{1}H experiments, 32k points of each of the 128 complex FIDs were acquired using a spectral width of 9 kHz (18.00 ppm) and a relaxation delay of 12 s.

The COSY experiments were acquired using a SW of 2.75 kHz (5.5 ppm) on both dimensions, 2K data points, 32 scans, 256 increments and a relaxation delay of 2 s.

The ^{1}H-^{13}C HSQC spectra were recorded using a SW of 5 kHz (10 ppm) (^{1}H) and 80 kHz (160 ppm) (^{13}C), 1K datapoints, 40 scans and 256 experiments with a relaxation delay of 2 s and a value of $^{1}J_{C\text{-}H}$ of 150Hz, while the ^{1}H-^{13}C HSQC-DEPT spectra were recorded using a SW of 4 kHz (8.00 ppm) (^{1}H) and 70 kHz (140 ppm) (^{13}C), 2K datapoints, 32 scans and 256 experiments with a relaxation delay of 2 s and a value of $^{1}J_{C\text{-}H}$ of 150Hz. The metabolites were assigned based on the 1D, 2D homo and heteronuclear correlation NMR spectra and by comparison with published data (Deshmuks et al., 2003; Ingallina et al., 2020; Wishart et al., 2022).

2.7. Statistical analysis

All extractions and analyses were performed in triplicates and data were expressed as average ± standard deviation. The results were analyzed by the ANOVA bootstrap, Duncan (SPSS 25, IBM, New York, NY, USA).

3. Results and Discussion

3.1. Set up of the analytical method to identify all-tomatine

The HPLC spectrum (Fig. S1-a) of the α-tomatine standard showed two peaks attributable to dehydro-tomatine and α-tomatine (retention times, RT of 13.3 min and 13.7 min, respectively) (Kozukue et al., 2004; Friedman et al., 2009; Taveira et al., 2012; Tamasi et al., 2019) usually considered together as α-tomatine form. Similarly, the tomatidine standard (Fig. S1-b) had two peaks at RT = 16.9 and 22.4 min recognized to the tomatidinedol and tomatidine (Taveira et al., 2012). Moreover, both spectra showed other peaks (RT = 7.8 - 12 min and RT = 22 - 26 min for the α-tomatine and tomatidine standards, respectively) already described by other authors (Friedman & Levin, 1998; Kozukue et al., 2004) but not better identified.

The correct attribution of the peaks was confirmed by the results of the acid hydrolysis of α-tomatine, which determined the rupture of the bonds between sugars and alkaloid portion (Fig. S2).

3.2 Extraction of the all-tomatine applying literature methods.

The method proposed by Taveira et al. (2012) to extract the leaves, stems and green fruits gave tomatine recovery comparable with those reported in this work, but with some difference on α-tomatine:tomatidine ratio. Several factors affected the concentration of the all-tomatine and the different qualitative composition of the extract. The main variable is the fruit stage of maturation and size, where the tomatine level decreases with the ripening and increase of fruit size (Friedman, 2002). These factors also can influence the formation of various tomatine isomers and intermediate forms that may be generated during plant metabolism. Among these the best known are the hydrolysis of sugars and ripening processes that lead to the formation or degradation of molecules structurally similar to α-tomatine (Friedman, 2002). Also, enzymes synthesized during fruit maturation can cause structural changes of dehydro-tomatine and α-tomatine isomers and transform them into dehydro-esculoside A and esculoside A, respectively, occurring through several steps which involve hydroxylations, acetylations and glycosylations (Friedman & Levin, 1998; Kozukue et al., 2004; Taveira et al., 2012; Zhao et al., 2021).

A higher extraction recovery of all-tomatine was achieved without SPE purification process on leaves (+60% of yield with SPE) and green fruits (+40% of yield with SPE), while no significant difference was found for the stems (Table 1). The SPE is considered a fast and effective purification system based on the different affinity of molecules for the chosen adsorbent solid phase (Friedman et al., 1994; Väänänen et al., 2000; Taveira et al., 2012; Caprioli et al., 2014). The SCX adsorbent phase employed in this study, containing silica with aliphatic groups of sulfonic acid on its surface, determined a different affinity of the extract's molecules based on their hydrophilicity degree. The

effect of the SPE purification on the extract was attributable to the interaction between the resin and both all-tomatine and no-tomatine fractions, due to their chemical characteristic (linkage site available). The more in-depth investigation of the results highlighted that the main all-tomatine retained fraction are the α-tomatine, probably due to resin-sugar linkages that were halved compared with those obtained without SPE, though no significant changes occurred for the tomatidine. The rate of α-tomatine retention, being linked not only to its concentration but also to the presence of other molecules involved in the saturation of the SCX linkage sites, was unfortunately not predictable and caused the loss of significant amount of the molecules of interest.

The second extraction method (Friedman et al., 2009) gave significantly lower yield starting from the same samples (Table 1). The absence of all-tomatine in the washing water of the precipitation step identified the extraction phase as being less efficient compared to the method of Taveira et al. (2012), while the purification showed very good result in term of all-tomatine pureness degree (Friedman et al., 2009) on all tested feedstock, except the leaves (Table 1). The use of diluted acetic acid and the short time of the extraction are aspects that reduce the cell wall permeability and the extraction yield. Moreover the washing with HCl, after the extraction, can affect the α-tomatine to obtain other form partially hydrolyzed (Friedman & Levin, 1998; Friedman, 2002).

The results of the extraction procedures applied to the tomato plant residues, highlighted the best performance for the extraction done with the acetic acid, moreover the precipitation with ammonia was better in term of purification and avoided loss of all-tomatine Therefore the two steps were mixed in the proposed method and tested by using the tomato waste as biomass.

3.4 Application of the proposed method to the tomato industry residues.

The application of the proposed method to the tomato industry residues gave all-tomatine recovery in the same range (0.64-6.66 mg g^{-1} DM biomass) of the literature (Taveira et al., 2012). The starting feedstocks SL1 and SL2 showed a similar concentration of α-tomatine and tomatidine, while the glycosylate form was more abundant than the aglycate one for the GT (α-tomatine:tomatidine of 17.6:1 and 3:1 for GT1 and GT2, respectively) (Table 1). The GT was made exclusively by the green fruits, that are very rich in α-tomatine which gets progressively degraded during the maturation of the fruit (Tamasi et al., 2019). A general reduction of the all-tomatine content occurred during the season of the cannery industry production (time of sampling 1 and 2) in particular, in the case of GT for the α-tomatine content (Table 1). The particular reason behind the change is not readily identifiable since several environmental and mechanical external factors such as crop management, light wavelength, damages by insects or postharvest conditions influences the all-tomatine cycle (Milner et al., 2011; Koh et al., 2013).

The extracts were in the range of 4.5 to 12.42 mg g^{-1} DM biomass, with the highest purity in terms of all-tomatine content found in SL1 and GT1 (740 and 540 mg g^{-1} DM E, respectively). At the second time of sampling, apart from the above-mentioned lower all-tomatine content, there was a higher presence of no-tomatine molecule susceptible to the ammonia precipitation that diluted the all-tomatine concentration, potentially affecting the bioactivity of the whole extract.

While α-tomatine has been considered the main active fraction of the whole class (Tamasi et al., 2019), also tomatidine and other SGA molecules characterized by a different number and composition on sugars were recognized as bioactives (Pingulkar & Kiran, 2001; Friedman, 2002; Woods et al., 2004; Simons et al., 2006; Friedman, 2013; Bailly, 2021; Zhao et al., 2021).

The use of an NMR approach allowed a more complete characterization of the SL1 extract and an attempt of identification of the other compounds, different from all-tomatine, found in the standard.

The ^{1}H NMR and HSQC spectra of the all-tomatine standard (Fig. S3, S4, S5, S6) showed significant signal difference for the protons H24, H23 and H25 that were thus selected as markers to discriminate tomatidine and the α-tomatine.

The ^{1}H NMR of the extract (Fig. 1) had several peaks generated by the presence of more molecules that, although not completely interpretable, confirmed the presence of other forms of tomatine. In addition, the detection of a signal similar to the one generated by the ^{4}Gal of tomatine, but slightly different in chemical shift, in the region 3.9-4.5 ppm can be ascribed to a tomatine-like species (Fig. 1).

The HSQC (Fig. 2) interpretation estimated the ratio of 3:2 between glycosylated:aglycate all-tomatine form, due only in part (60%) to the α-tomatine identified by the typical Gal: 2 Glu: Xyl ratio. For the remaining 40%, the signals of the anomeric region highlighted spectral profiles compatible with three different chemical structures of the glycosylate portion: i) presence of a trisaccharide Gal-Glu-Glu and the two new anomeric signals detected in the HSQC (Fig. 1) are the glucose signals that shift due to the absence of xylose directly linked; ii) presence of a tetrasaccharide containing galactose, probably glucose, and some other saccharide which anomeric proton resonate near that of the β-glucose; iii) presence of a trisaccharide Gal-Glu-Glu with some chemical changes. Indeed, in the region between 5.00-5.5 ppm of the proton spectrum and 120-135 ppm of ^{13}C in the HSQC map, some signals generated by unsaturated chain can be attributed to the glycosidic part of other tomatine-like species or to different molecules.

The quantification done with the HPLC (Table 1) confirmed the α-tomatine:tomatidine ratio (0.9:1) found by NMR approach and allowed to identify peaks not recognized before in the HPLC spectra (Fig. S1) in particular these located between the α-tomatine and tomatidine (RT = 18.19-21.82 min). The calculation of the concentrations of the tomatine-like molecules, done using the α-tomatine standard as reference, gave a glycosylate:aglycate ratio very similar to that of the NMR, therefore describing a higher pureness degree of all-tomatine in the SL1 extract (all-tomatine = 864 mg g^{-1} DM E) compared to the one previously calculated. The same method was applied to the HPLC spectra of the other extracts to obtain a general increase to 661, 321 and 210 mg g^{-1} DM E for GT1, SL2 and GT2 respectively. The analytical characterizations (Fig. S1, 1, 2) identified the other molecules as fatty acids and hydroxyl fatty acids (Deshmuks et al., 2003; Lerma-García et al., 2011;) both being components of cutine, the polymer of the cuticle layer found in fruits, stems, and leaves with protection functionality. Their presence in the extract despite their limited solubility in hydrophilic solutions can be explained by considering the proximity between the cuticle and the cell wall and the presence of bonds among those molecules. The recent theory on morphological structure of the cuticle highlighted the closeness between cutine and cell wall in portions embedded in the cuticle thickness. In particular, strong interactions were described between pectine and hemicellulose for which covalent ester/hydrophobic bonds have been described (Glenn et al., 2020).

3.4.1 Characterization of the residues fraction of the all-tomatine extraction system

The process to obtain the all-tomatine extract gave two discharge fractions at the end of the extraction and precipitation steps, respectively (Table 2). The main fraction was obtained after the extraction phase and corresponded to the 68.06 ± 0.45 % DM and 51.78±2.56% DM as average for the SL and GT, respectively (Table 2). Compared to the starting samples, the main reductions were registered for soluble content (CS) in GT and hemicellulose in SL. The CS composition included

miscellaneous molecules such as organic acids, proteins, sugars, polyphenols as well as the all-tomatine that were accumulated in the vacuole and cytoplasm (Roddick, 1974). In tomato plants, the hemicelluloses have an atypical chemical composition and a soluble nature (Broxterman & Schols, 2018) both of these characteristics enhanced their hydrolysis by acetic acid and the solubilization of the detached portion (Szymańska-Chargot et al., 2017; Hu et al., 2018; Yang et al., 2018). It is known how external factors (i.e. seasonality) can affect the vegetal tissue composition (Pardini et al., 2021): in the case of the SL the main difference regards the hemicellulose vs. cellulose ratio, while for the GT it concerns CS. The intensity and nature of these structural changes affected the extractability of the different vegetal biomass components; by using a mass balance approach, the amount of each macromolecular fraction present in the extract was quantified. For GT, the CS was the fraction more present in the extract (66% and 83% for GT1 and GT2, respectively), while for SL there was a more balanced amount of CS and hemicellulose (43%:57% and 42%: 35% for CS:hemicellulose for SL1 and SL2, respectively). Moreover, a partial extraction of the cellulose occurred for both biomasses sampled at time 2 (cellulose extracted: 22% and 3% for SL2 and GT2, respectively). The cellulose was the only insoluble component of the tomato cell wall; despite its good reactivity due to the microfibrilles, its big size and low crystallinity degree caused it to be the lesser extractable fraction, possibly due to its insoluble characteristic and/or to the scarce power of the extraction solution. With reference to the Exh composition, the significant extraction of the more biodegradable CS improved the recalcitrance of the biomass that, together with the good fiber content, suggested a possible successive re-use as cultivation substrate.

The successive precipitation with ammonia selected 1.04 ± 0.14 and 0.84 ± 0.5% DM as average for SL and GT respectively showing a great selectivity (extracts = 3% and 1.7 % DM of the amount treated with ammonia) but causing the production of a large quantity of wastewater with high pH (around 11) and ammonia content that limited successive employment. On the other hand, the

possibility to regain this fraction is an interesting aspect of the process in the perspective of minimizing waste.

With the aim of reducing ammonia concentration and pH, a dialysis approach was tried. The washing of the fraction under dialysis lowered the starting value to reach pH = 9-9.5 (Table 3). The NDF had a significant carbon content, and the N was present above all in organic forms (Table 3). The exploratory ^{1}H NMR spectra of NDF solubilized in D_2O and in d-methanol (spectra not reported) showed the presence of sugars and aliphatic compounds, principally acetic acid and lactic acid, plus lesser intense polipeptidic and aromatic profile. Unfortunately, the dialysis caused the loss of the 45.9%, 52.66%, 55.67%, and 61.4% of the starting DM therefore alternative treatment system will be considered in the perspective of maintaining a high recovery.

4. Conclusion

The described process, allowed to extract and concentrate molecules with a high biological value starting from waste material, as well as implementing a cascade production to further minimize waste produced along the process, will allow a more environmentally-sustainable use of all-tomatines, possibly in a circular economy approach in which they can be employed as biopesticides to act against biological stressors of tomato. The extraction with acetic acid and the successive ammonia precipitation gave the best extraction performance to produce fraction rich in all-tomatine from the residues of the tomato cannery industry. The extract had different all-tomatine content and qualitative characteristics depending on the composition of the feedstock (leaves, green fruits, and/or stems) and by the seasonality. The process allowed the valorisation of the solvent-free by-product obtained after acetic acid extraction to be re-used as cultivation substrate. The dialysis treatment of the ammonia-rich wastewater obtained as a by-product of the

precipitation step, allowed only a partial recovery of the organic matter fraction, thus alternative systems will be evaluated. The best process performance in term of all-tomatine and mass balance recovery has been obtained using the stem+leaves (SL1) as feedstock (yield of the extract: 10.8 mg g^{-1} dry DM; all-tomatine pureness: 864 mg g^{-1} DM extract; biomass recovery: 68% DM starting biomass).

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Conflicts of interest

The authors declare no conflict of interest.

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Tables

Table 1. Extraction yields of all-tomatine and fraction obtained with the method by Taveira et al. (2012), Friedman et al. (2009) and that proposed in this work

		α-tomatine[a]	tomatidine[b]	all-tomatine	Extract	α-tomatine[a]	tomatidine[b]	All-tomatine
		mg g^{-1} DM biomass				mg mg^{-1} DM Extract		
Taveira et al. (2012)	Leaves	1.7±0.21*,[γ]a	0.42±0.02*a	2.1±0.24*a	-	-	-	-
		5.41±0.74**b	0.37±0.002**a	5.81±0.74**b	-	-	-	-
	Stem	1.1±0.19*a	0.45±0.02*a	1.54±0.18*	-	-	-	-
		1.58±0.01**ab	0.37±0.01**a	1.95±0.01**ab	-	-	-	-
	Green fruit	0.72±0.04*a	0.35±0.06*a	1.07±0.1a	-	-	-	-

		1.45±0.2**b	0.24±0.06**a	1.69±0.27**a	-	-	-	-
Friedman et al. (2009)	Leaves	0.3±0.2	0.14±0.07	0.43±	35.3±0.6	10.5±	4.66±4	15.16±
	Stems	0.83±0.17	0.10±0.003	0.93±0.2	5.14±0.01	427±50.3	51.7±1.8	479±68
	Green fruit	0.69±0.11	-	0.69±0.11	9±0.02	622±102	-	622±102
Method proposed	SL1	3.4±0.5	4.6±0.13	8.01±0.37	10.8±0.4	310±40	430±10	740±30
	GT1	6.66±0.9	0.4±0.09	7.062±0.9	12.4±4	530±70	30±8	570±80
	SL2	1.52±0.1	1.55±0.2	3.07±0.1	9.6±2	158±10	160±19	318±8.8
	GT2	0.64±0.02	0.21±0.01	0.86±0.19	4.5±1	142.8±5.8	48.2±12.5	191±18

[a]α-tomatine+Dehydro-tomatine, [b] tomadine+tomatidinedol

* samples purified with SPE

** sample not purified with SPE

[γ] The same sample treated or not with SPE (Taveria et al., 2012) followed by the same letter are not statistically different (ANOVA bootstrap, Duncan post-test).

Table 2. Tomato waste composition and chemical characterization of the exhausted of the extraction step

Sample	Composition	% weight/weight	DM (% WW)	Soluble contents	Hemicellulose	Cellulose	ADL	Insoluble Ash
				(% DM)				
SL 1	Leaves and Stems	78.35	14.31	43.35±0.8	30.27±0.43	13.51±0.47	9.83±0.35	3.05±0.26
	Green tomato	5.53						
	Red tomato	16.12						
GT 1	Green tomato	80	6.4	49.75±0.96	26.55±1.26	11.59±2.11	10.54±2.25	1.57±0.01
	Red tomato	20						
SL 2	Leaves and Stems	66.58	16.65±2.47	43.65±2.07	21.97±1.75	23.06±0.07	8.62±0.46	2.70±0.1
	Green tomato	24.42						
	Red tomato	9						
GT 2	Green tomato	86	6.18±0.22	62.50±1.24	20.56±1.14	9.99±1.01	5.71±0.74	0.84±0.01
	Red tomato	14						

Exh_SL 1	43.48±0.11	17.92±0.39	19.92±0.14	15.05±0.15	3.63±0.01
Exh_GT 1	40.95±0.03	22.79±0.83	17.49±1.28	16.91±1.29	1.86±0.09
Exh_SL 2	46.09±1.05	17.14±0.13	24.29±0.22	9.87±1.10	2.61±0.09
Exh_GT 2	37.87±2.57	26.32±2.40	16.98±1.05	16.88±1.34	1.95±0.08

Table 3. Chemical characterization of the NDF.

		NDF_SL1	NDF_GT1	NDF_SL2	NDF_GT2
ash	%DM	13.8±2.3	11.3±1.1	9.65±0.98	15.13±1.5
C organic		501±38	521±19	535±63	493±12
total nitrogen		63.3±2.1	47.2±1.4	86.3±1.7	76.1±2.2
NH_4-N	mg g^{-1}	23.8±2.2	22.1±2.04	31.9±0.37	29±0.51
NO_3-N		0.49±0.06	1.02±0.07	0.46±0.01	1.24±0.09
Organic-N*		39.01	24.08	53.94	45.86

* organic N calculated as (total nitrogen- NH_4-N- NO_3-N)

Caption figures

Fig. 1 ^{1}H- NMR spectrum of SL1 extract. On the left is reported the enlargement of the spectral region 3.9-4.5ppm.

Fig. 2. a) HSQC spectra of SL1 with an enlargement of the regions (F1:15-50 ppm; F2:0.5-2.7 ppm), and (F1:90-145 ppm; F2:4.0-6.0 ppm) signal marked with an asterisk (*) were alternative to those of the α-tomatine. b) HSQC spectra of SL1 with an enlargement of the region (F1:15-50 ppm; F2:0.5-2.7 ppm) containing cutin signals.

Figures

a)

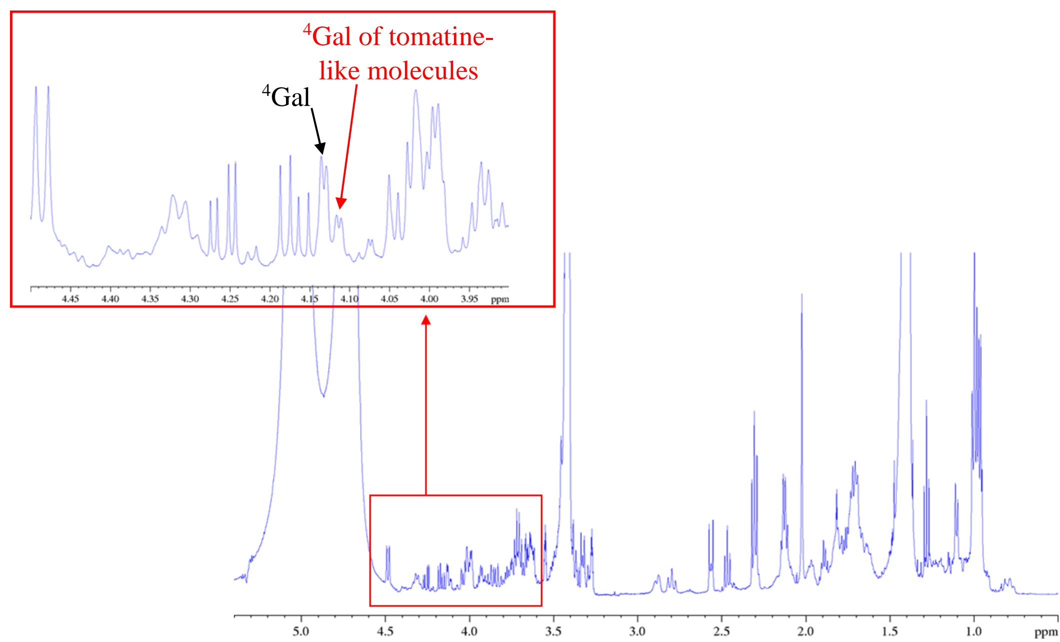
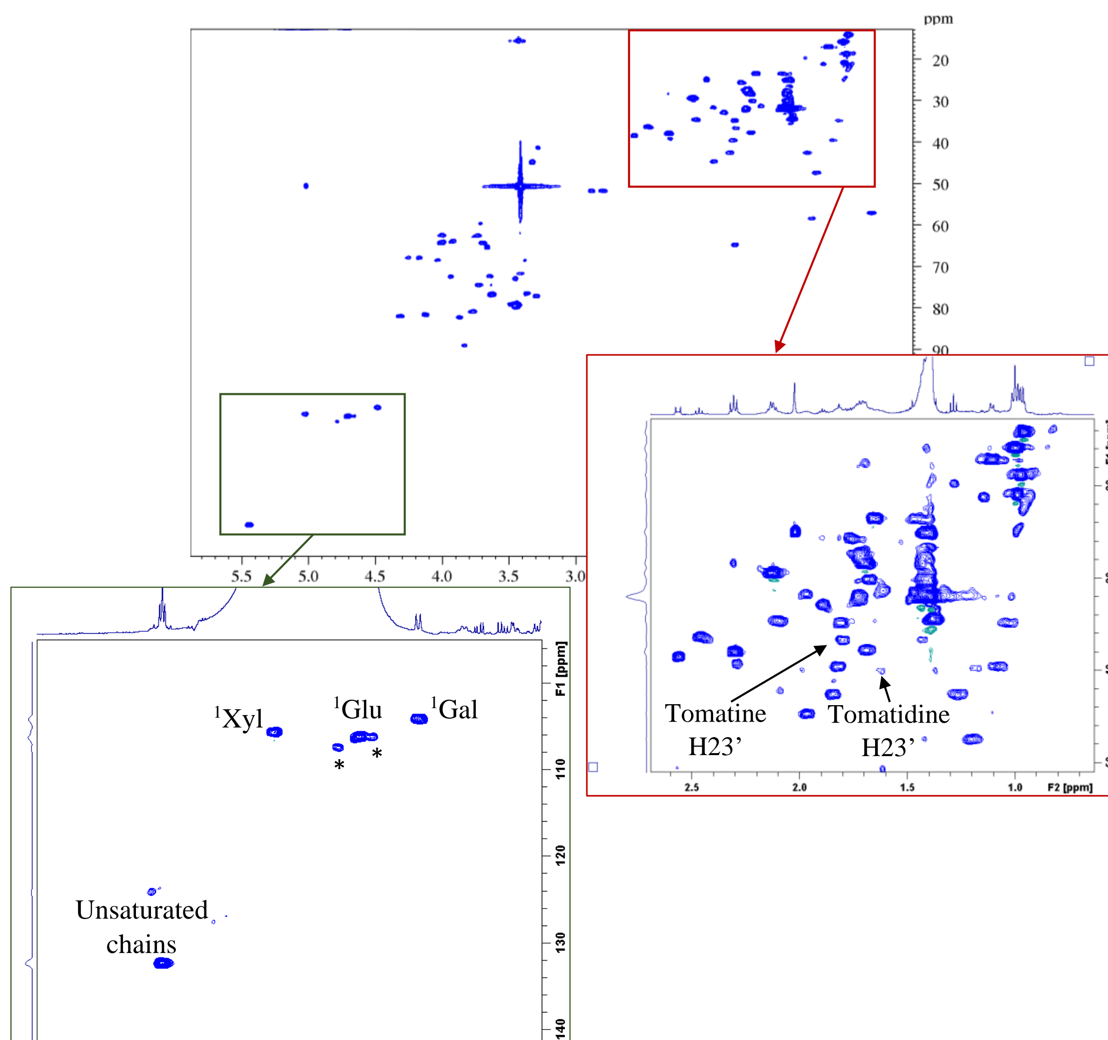


Fig. 1

a)



b)

Symbol	*Structure*
A1	**CH$_3$**-CH$_2$-CH$_2$-R
A2	CH$_3$-CH$_2$-**(CH$_2$)$_n$**-CH$_2$-R
B1	R-CH$_2$-CH$_2$-CH$_2$-CO-O-**CH$_2$**-CH$_2$-CH$_2$-R
B2	R-CH$_2$-CH$_2$-CH$_2$-CO-O-CH$_2$-**CH$_2$**-CH$_2$-R
B3	R-CH$_2$-CH$_2$-CH$_2$-CO-O-CH$_2$-CH$_2$-**CH$_2$**-R
C1	R-CH$_2$-CH$_2$-**CH$_2$**-CO-O-CH$_2$-CH$_2$-CH$_2$-R
C2	R-CH$_2$-**CH$_2$**-CH$_2$-CO-O-CH$_2$-CH$_2$-CH$_2$-R
D1	R-CH$_2$-CH$_2$-**CH$_2$**-OH
D2	R-CH$_2$-**CH$_2$**-CH$_2$-OH
D3	R-**CH$_2$**-CH$_2$-CH$_2$-OH
E1	R-CH$_2$-CH$_2$-**CH**OH-CH$_2$-CH$_2$-R
E2	R-CH$_2$-**CH$_2$**-CHOH-**CH$_2$**-CH$_2$-R
E3	R-**CH$_2$**-CH$_2$-CHOH-CH$_2$-**CH$_2$**-R
F1	R-CH$_2$-**CH$_2$**-CO$_2$H
F2	R-**CH$_2$**-CH$_2$-CO$_2$H
G	R-CH$_2$-CH$_2$-**CH**(CH$_2$-CH$_2$-R)-CO-O-R
H	R-CH$_2$-CO-O-**CH**(CH$_2$-R'')-CH$_2$-R'
L	R-CH$_2$-**CH**=CH-CH$_2$-CH=CH-R

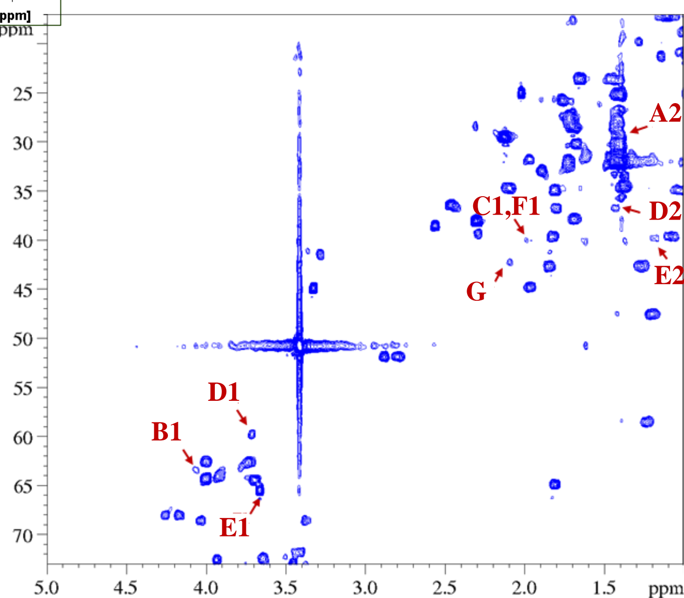


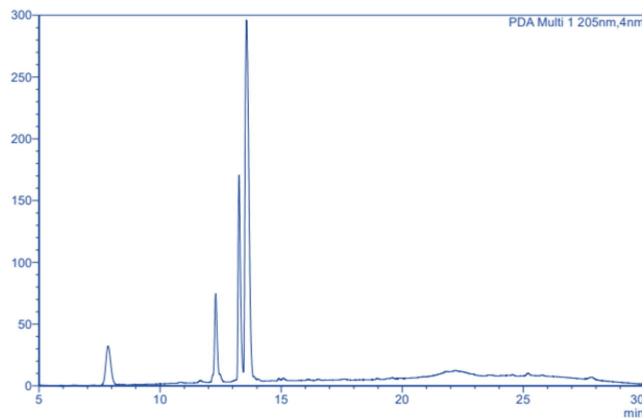
Fig. 2

Supporting Information

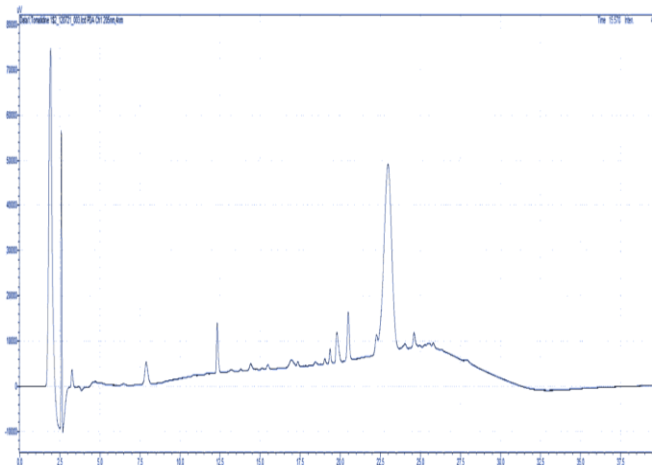
Development of a cascade production system finalized to the extraction of all-tomatine-rich fraction using the tomato cannery waste as feedstock

Parisa Abbasi-Parizad[a], Rosachiara Antonia Salvino[b], Alessandro Passera[a], Alessia Regina Vera Follador[a], Cesare Cosentino[b], Costanza Jucker[c], Sara Savoldelli[c], Jacopo Bacenetti[d], Paola Casati[a], Barbara Scaglia[a,*].

a)



b)



c)

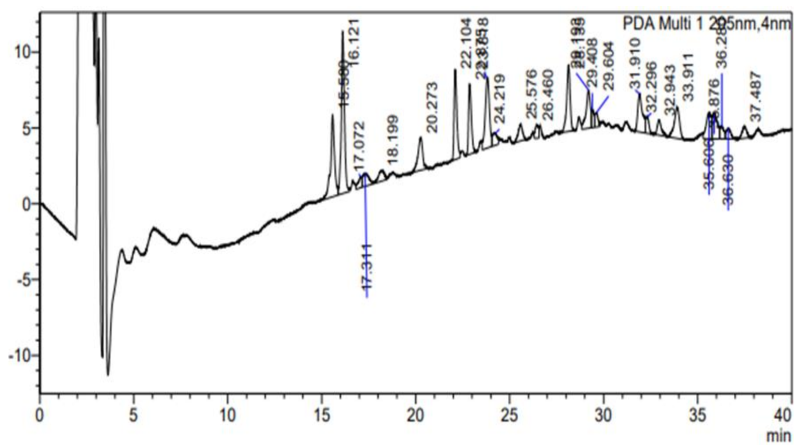


Fig. S1: Spectra HPLC of a) α-tomatine, b) tomatidine standards and c) SL1 extract

S1. Test of acid hydrolysis of the α-tomatine standard

S1.1. Method

Hydrolysis experiment on α-tomatine standard was carried out as described by Kuronen et al. (1999). Briefly, 2 mg of α-tomatine standard was dissolved in 1 mL methanol in which 0.25 mL HCl (25%) was added, and the mixture was heated at 80 °C with stirring for 2 hours (sampling at 1h intervals). After 1 h and at the end of the experiment the ammonia solution (25% w/w) was added to get the pH up to 10 in order to precipitate the all-tomatine. Then 1 mL of dichloromethane was added two times. The aqueous phase was successively separated by adding Na_2SO_4 salt and the dichloromethane solution of each experiment (1h, 2h) was brought to dryness in a vacuum oven at 40 °C. The residues were then re-suspended in 1 mL methanol and chemically characterized as reported before. The experiments were performed in triplicates.

S1.2. Result

After 1 h of treatment, α-tomatine and dehydrotomatine disappeared while a big peak with the same RT of the tomatidine and tomatidinedol became visible (Fig. S2). Moreover, two other smaller peaks before and after the aglycones ones (RT around 20 and 25 min, respectively) came into view, probably detecting alternative typology of all-tomatine originated by the chemical reactions. After 2 h of treatment, all peaks disappeared except for the unknown peaks in the α-tomatine standard, that were attributed to molecules that did not belong in the group of all-tomatine.

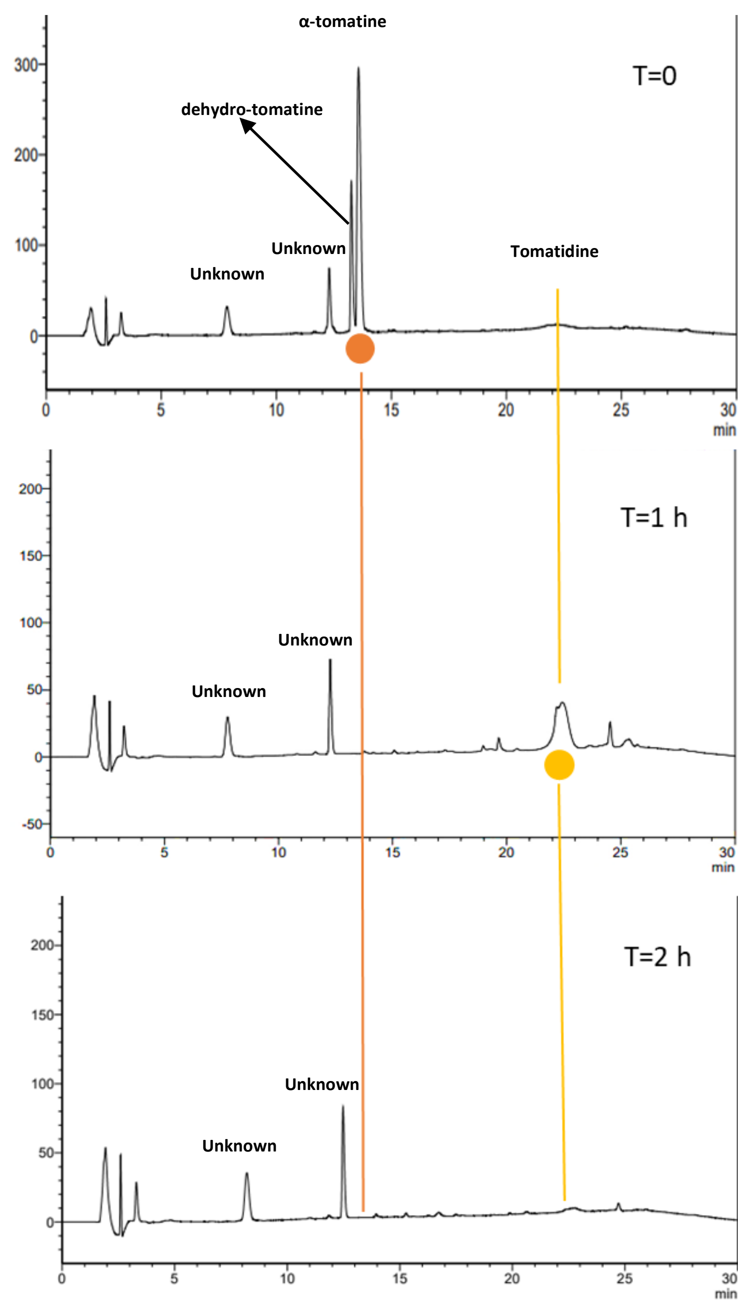
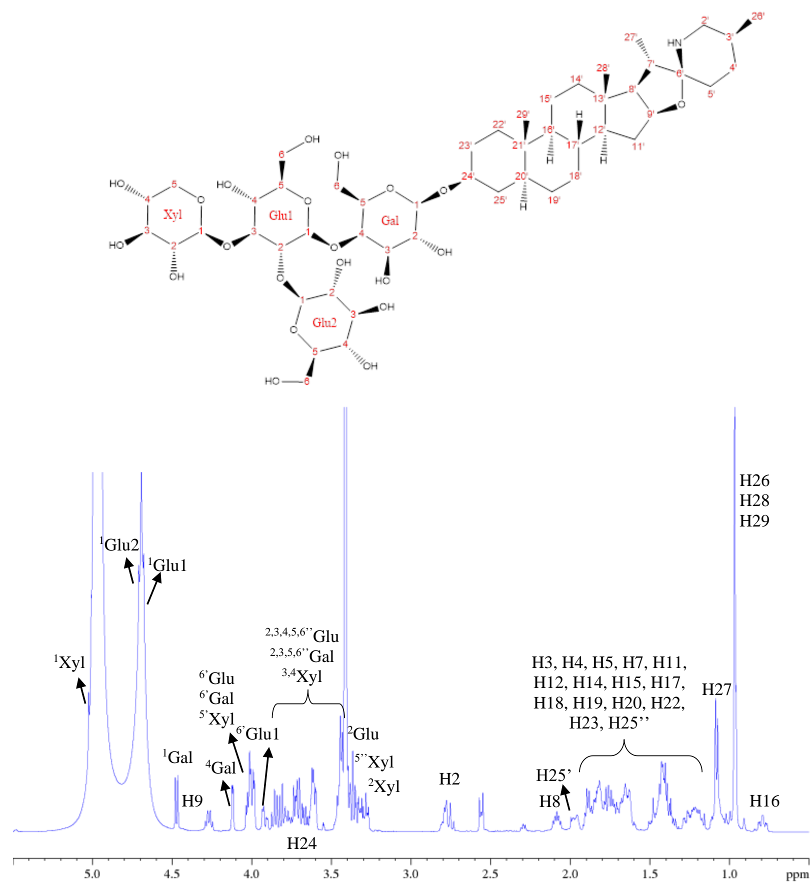


Fig. S2. Kinetic of the acid hydrolysis process of the α-tomatine standard (α-tomatine (orange arrow); tomatidine (yellow arrow)).

a)



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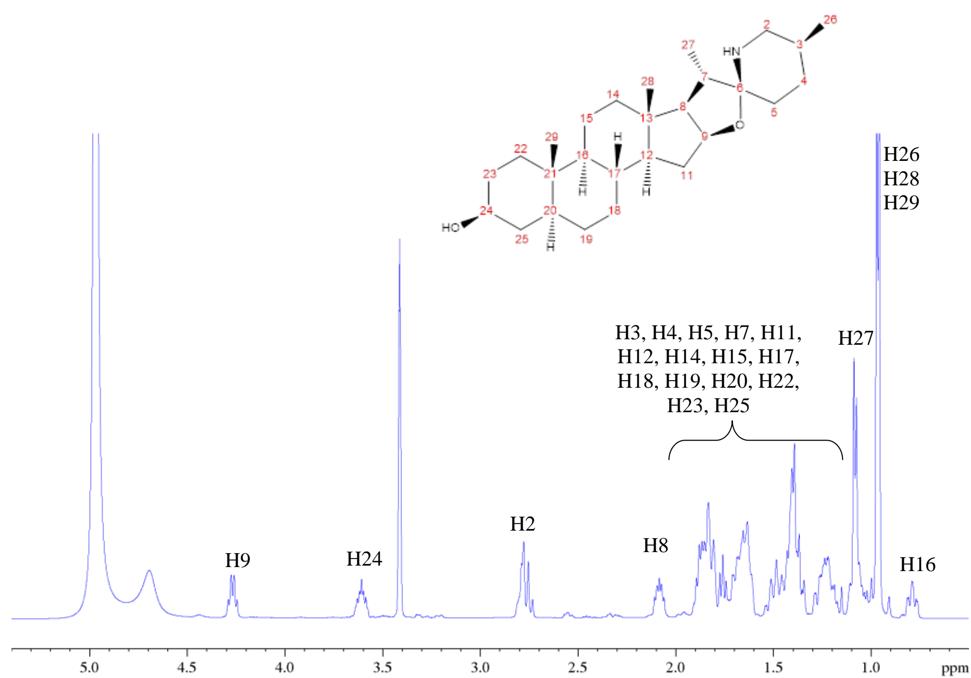
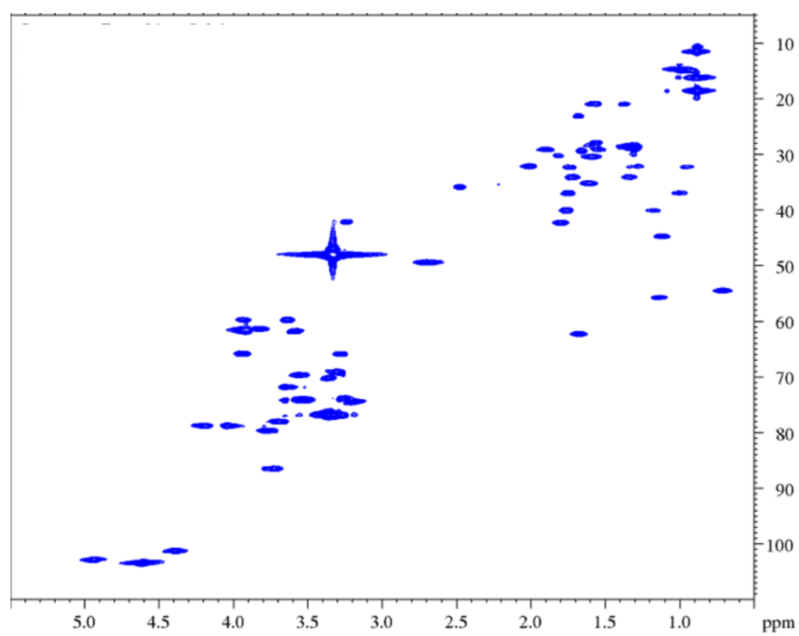


Fig. S3. ^{1}H NMR spectra of a) tomatine and b) tomatidine standards dissolved in Methanol- d_4.

a)



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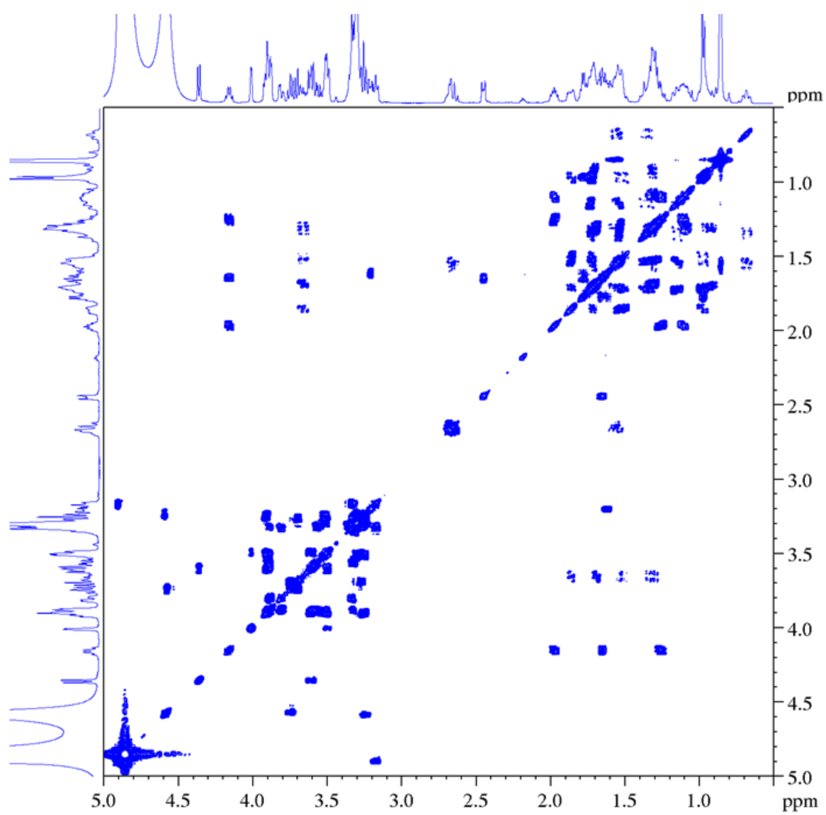


C)

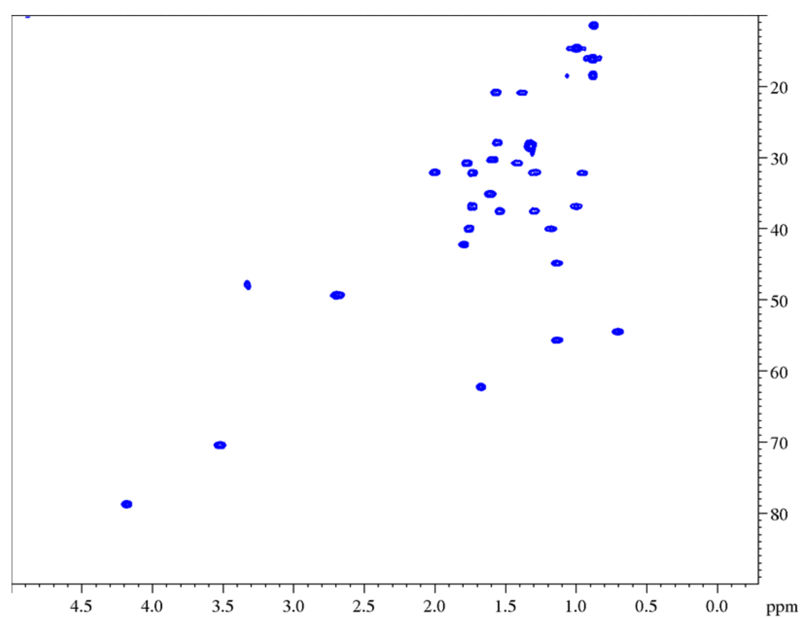
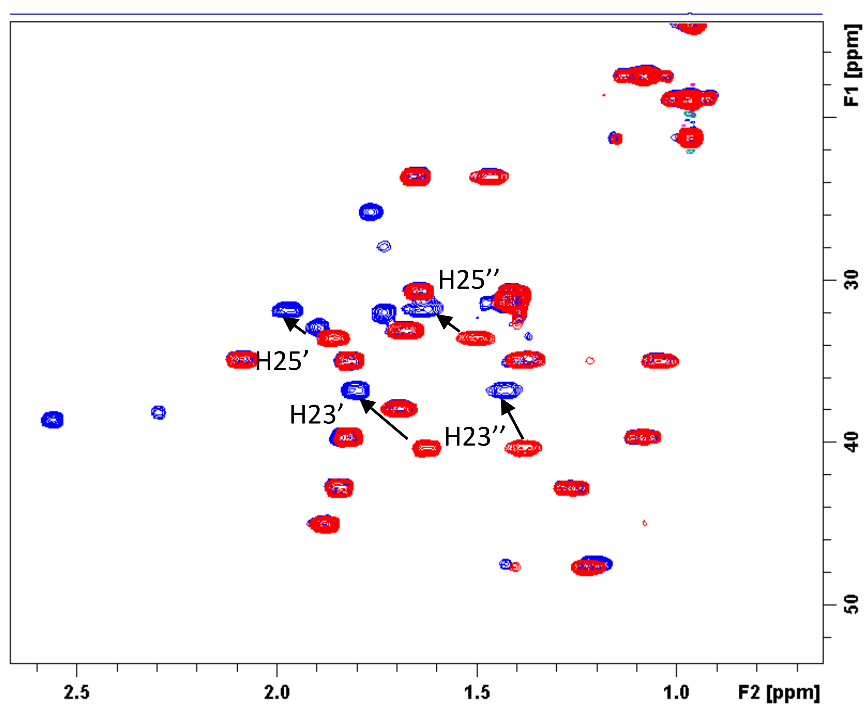


Fig. S4. a) HSQC spectrum of α-tomatine standard, b) *COSY spectrum of α-tomatine dissolved in Methanol- d_4.*and c) HSQC spectrum tomatidine standard dissolved in Methanol- d_4.



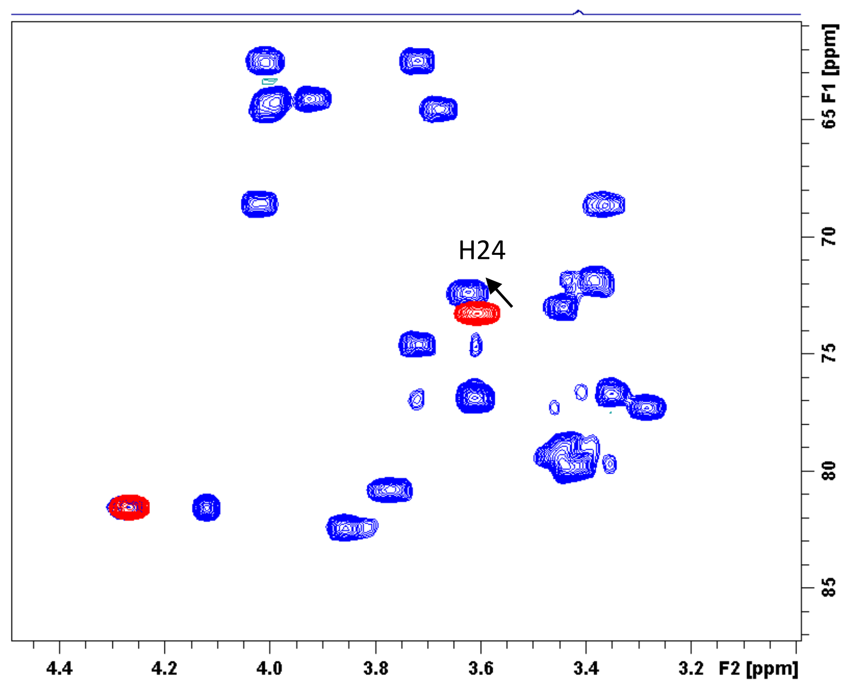
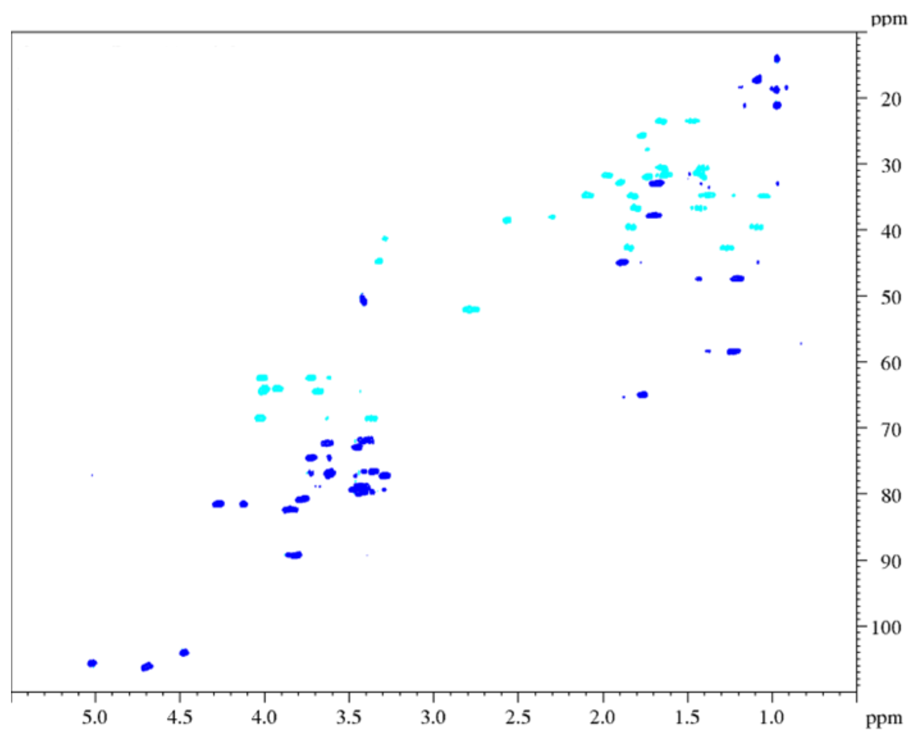


Fig. S5. HSQC spectra of α-tomatine in blue and tomatidine standards in red. Top: F1:15-53ppm – F2:0.6-2.7ppm; Bottom: F1:60-87ppm – F2:3.0-4.5ppm.

a)



b)

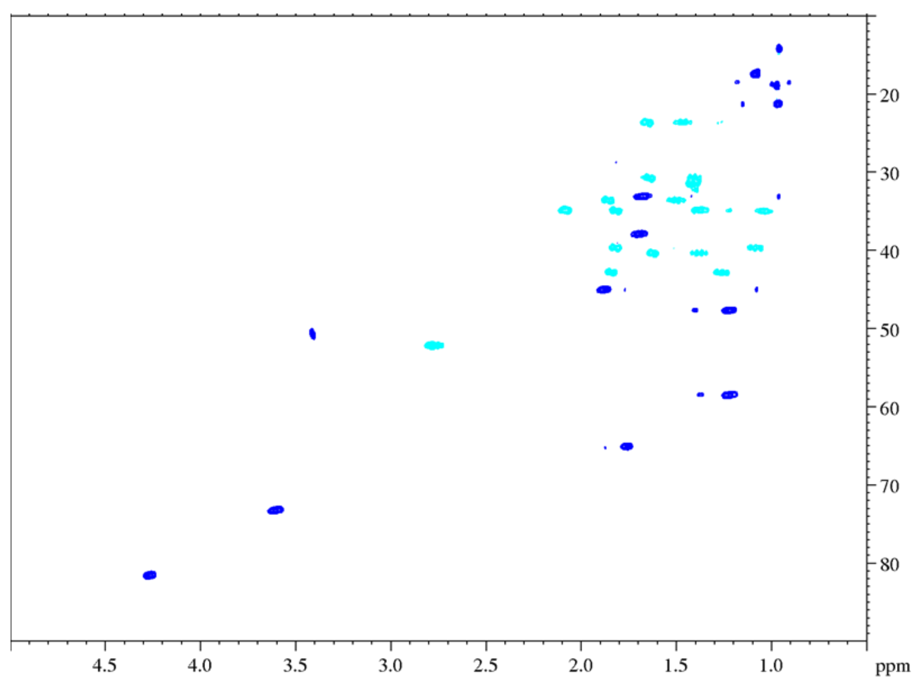


Fig. S6. HSQC-DEPT spectrum of a) α-tomatine, b) tomatidine standard dissolved in Methanol- d_4.