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Comparison of gastrointestinal stability of isothiocyanates from *Tropaeolum Majus* L. *Altum* using *in vitro* and *ex vivo* digestion methods

Ivana Vrca[1*], Franko Burčul[1], Ivica Blažević[1], Andre Bratanić[2], Tea Bilušić[1]

[1]*University of Split, Faculty of Chemistry and Technology, Ruđera Boškovića 35, 21 000 Split, Croatia*
[2]*Division of Gastroenterology and Hepatology, University Hospital Split, Spinčićeva 1, 21000 Split, Croatia*

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ABSTRACT

Tropaeolum majus L. is an annual herbaceous plant and a member of the Tropaeolaceae family, which belongs to the Brassicales order. It is an excellent source of flavonoids, carotenoids, phenolic acids, vitamin C, and it is a plant that contains the glucosinolate glucotropaeolin. The major degradation product of glucotropaeolin is benzyl isothiocyanate which is known for its various biological activities. In this study, an essential oil was isolated from the seeds of the *Tropaeolum majus* L. *altum* plant by microwave-assisted distillation and analysed using the GC-MS technique. Two compounds were identified, benzyl isothiocyanate as the major one (97.81%), and 2-phenylacetonitrile as a minor one (0.80%).

Tropaeolum majus L. *altum* essential oil and pure benzyl isothiocyanate were then submitted to the two-phase *in vitro* and *ex vivo* digestion simulations. The analysis performed by the GC-MS/MS technique showed greater stability of benzyl isothiocyanate from essential oil after *in vitro* (97.57%), and *ex vivo* (73.47%) gastric phases of the simulated digestion methods, compared to its stability after *in vitro* (71.17%) and *ex vivo* (54.90%) intestinal phases. A similar trend was shown for pure benzyl isothiocyanate.

Introduction

Tropaeolum majus L. (Indian cress) is a plant from the Tropaeolaceae family, which belongs to the Brassicales order. It is an ornamental, annual, fast-growing, bushy or branched (about 30 cm high), and tall or climbing plant (it can grow up to 90 cm) (Niizu and Rodriguez-Amaya, 2005). The subspecies of the *T. majus* plant differ according to the structure, size, and colour of the flower (Jakubczyk et al., 2018). Leaves and flowers are edible and they are used in dishes because of the peppery taste which is attributed to glucosinolates. Also, the plant is known for its ornamental and medicinal properties (Garzon and Wrolstad, 2009). Due to the presence of glucosinolates, it is considered a valuable plant because its degradation products (isothiocyanates) have a wide range of biological activities such as anticancer, antimicrobial, and anti-inflammatory activities (Wu et al., 2009, Romeo et al., 2018, Wagner et al., 2011). The antimicrobial activity of the *T. majus* can be attributed to benzyl isothiocyanate, which is the major degradation product of the glucosinolate glucotropaeolin (Fig. 1) (Bazylko et al., 2013).

Different parts of the *T. majus* are also used because of their antidiabetic and anti-obesity properties (Jurca et al., 2018). The stability of individual bioactive components found in food is not always known. The rate of stability of bioactive compounds affects their putative effect on the human health. The stability rate can be examined by *in vitro* digestion methods which usually consist of two phases - gastric and intestinal. Several factors, including sample characteristics, digestive enzyme activity, ion composition, digestion time, and pH levels have a significant impact on the results obtained by *in vitro* digestion experiments (Hur et al., 2001).

*Corresponding author E-mail: ivrca@ktf-split.hr

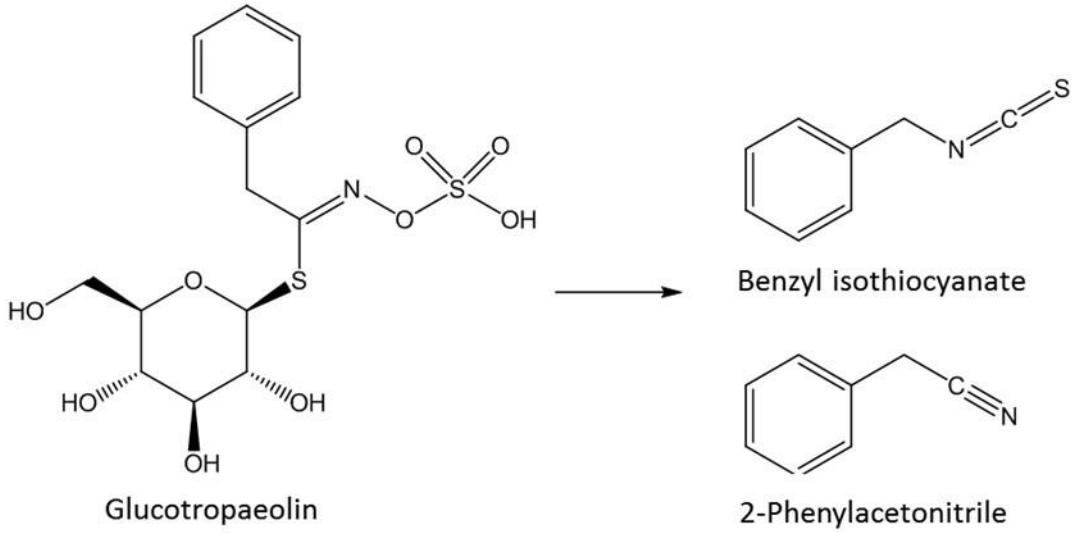

Fig. 1. Degradation of the glucosinolate glucotropaeolin

Generally, gastric digestion is simulated by acidifying a sample to a pH value close to that of stomach juice of a healthy adult (pH 2-3). The simulation of small intestine digestion, on the other hand, is performed by neutralizing the sample to pH 6.5–7.0, which is closest to the pH value of intestine juice of a healthy adult (Fernandez-Leon et al., 2017). According to available literature, differences in protein and peptide profiles were demonstrated when commercial digestive enzymes were compared with human digestive juices after a simulated digestion procedure. The enzymatic complexity of human digestive juices arises due to the presence of various enzymes, bile salts, and enzyme inhibitors involved in the process of food digestion (Furlund et al., 2012). Individual variations observed in human digestive enzymes are related to differences in enzymatic activity and volumes of gastrointestinal juices (Furlund et al., 2012). Gastric pH varies with age and the feeding period, which makes an *in vitro* digestion simulation challenging (Furlund et al., 2012). Scientific studies related to the positive impact of isothiocyanates on human health after consuming food containing glucosinolates are still insufficiently elucidated. The *in vitro* digestion method has shown a significant effect on the isothiocyanate content after digestion (Puangkam et al., 2017). Rodriguez-Hernandez et al. (2013) have proven that the isothiocyanate concentration decreases after the *in vitro* digestion method. According to Puangkam et al. (2017), the amount of phenethyl isothiocyanate was reduced by half after the *in vitro* digestion method.

The aim of this study was to determine the biologically active compounds obtained from the seeds of the *Tropaeolum majus* L. *altum* (TMA) plant by microwave-assisted distillation, and their stability rate after two-phase digestion simulations - an *in vitro* method based on the use of commercial digestive enzymes from the stomach and the small intestine (Brodkorb et al., 2019), and an *ex vivo* method with human digestive juices from healthy donors (Blažević et al., 2020a).

Materials and methods

Plant material

The seeds of the *Tropaeolum majus* L. *altum* (TMA) were commercially obtained from Marcon d.o.o., Novi Marof, and produced by Hortus sementi, Italy. For the extraction of essential oil from the seeds of the TMA, a sample was ground to a fine powder using a coffee grinder. Afterwards, the sample was soaked in distilled water, just before the microwave-assisted distillation (MAD).

Isolation of volatile compounds

The volatile compounds were isolated from the TMA seeds by MAD using an ETHOS X device (Milestone, Italy) and applying microwave power of 500 W. The plant material (100 g) was placed in a reactor inside a microwave oven (Fig. 2). The temperature was ca. 98 °C, and the extraction time was set to 30 min, with the additional 5 min required to cool the extraction system. After the MAD, the TMA essential oil, collected in the pentane trap, was dried with anhydrous sodium sulphate to remove any remaining water. The sample was stored in vials at -20 °C until further analysis.

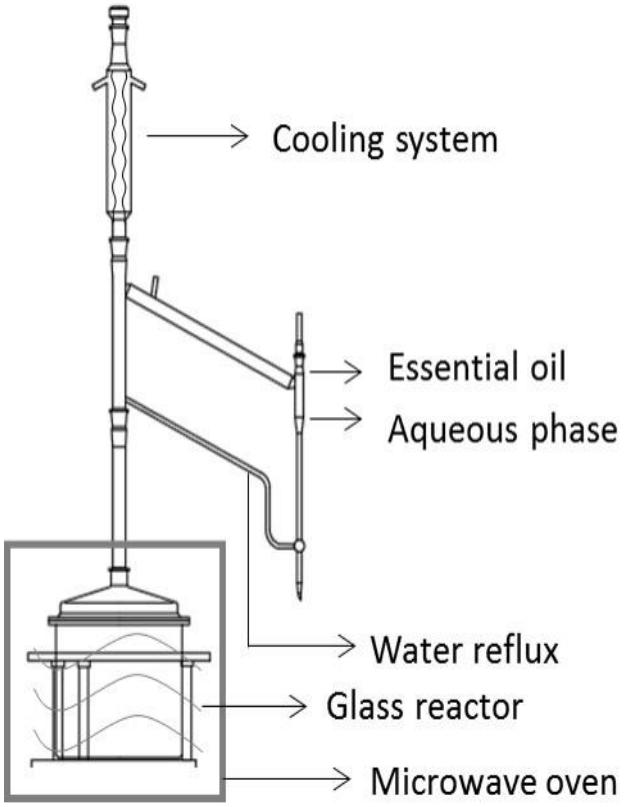

Fig. 2. Obtaining essential oil by microwave-assisted distillation (MAD)

The yield of essential oil was calculated according to Eq. 1:

$$\text{Yield (\%)} = (\text{mass}_{\text{of essential oil}} / \text{mass}_{\text{of plant material}}) \times 100\% \quad (1)$$

Gas chromatography-mass spectrometry (GC-MS)

A gas chromatograph (model 3900; Varian Inc., Lake Forest, CA, USA) equipped with a mass spectrometer (model 2100T; Varian Inc.) was used to identify volatile compounds in the essential oil from the seeds of the TMA plant. The sample was separated using a non-polar column VF-5MS (dimensions: 30 m long, 0.25 mm inner diameter, and 0.25 μm stationary phase layer thickness). The carrier gas was helium, and the flow rate was set at 1 mL min^{-1}. The inlet temperature was 250 °C, while the volume of the injected sample was 1 μL. The column temperature program was set at 60 °C for the first 3 minutes and then heated to 246 °C at a rate of 3 °C min^{-1}, and maintained for 25 min isothermally. The conditions set in the mass spectrometer were: ionization energy of 70 eV, ion source temperature of 200 °C, and a scanning range of 40-350 m/z units.

In vitro and ex vivo digestion methods

The two-phase *in vitro* digestion method involves simulated digestion in the stomach and the small intestine with commercial digestive enzymes, and a two-phase *ex vivo* digestion method with human digestive juices collected from healthy donors. The TMA essential oil and pure benzyl isothiocyanate compound (Sigma-Aldrich, Merck KgaA, USA) were prepared in 70% ethanol at a concentration of 2 mg/mL. The total volume of undigested samples and with digestive juices (human and commercial) was set at 1.5 mL. Prior to the addition of human gastric juice, the sample was adjusted to pH 2 using 1 M of HCl. Undigested samples were diluted with distilled water to 1.5 mL. The sample with human gastric juice consisted of 750 μL of TMA essential oil, 260 μL of gastric juice, and 490 μL of distilled water. The sample with human intestinal juice contained 750 μL of TMA essential oil, 650 μL of human intestinal juice and 100 μL of distilled water. The samples that contained human intestinal juices were in the range of the desired neutral pH value, so there was no need to neutralize them. The samples with commercial gastric enzymes consisted of the TMA essential oil and simulated gastric fluid containing RGE15 (Rabbit gastric extract, Lipolytech, France). The RGE15 extract contains gastric pepsin and lipase with no less than 500 U/mg of pepsin and 15 U/mg of lipase. The sample with commercial intestinal enzymes contained the TMA essential oil and simulated intestinal fluid, in which pancreatin and bile salts were dissolved (Sigma-Aldrich, Merck KgaA, USA) in a ratio 1:1, according to the method of Brodkorb et al. (2019) with modifications. Gastric and intestinal simulated digestions were conducted separately and not consecutively, in both *in vitro* and *ex vivo* simulations.

The simulation of digestion in the gastric and intestinal phase was performed in an incubator shaker (Thermo-TS-100, Biosan, Latvia) at the temperature of 37 °C and speed of 1200 rpm. The samples which contained gastric digestive enzymes at pH 2 were incubated for 60 minutes and the samples with intestinal digestive enzymes at pH 7 were incubated for 120 minutes. After incubation, the samples were placed on ice for 5 min to stop the enzymatic reaction. The samples were then centrifuged (10000 rpm; ThermoScientific mySpin 12, Thermo Fisher Scientific, Waltham, MA, USA) for 5 minutes at room temperature to separate the supernatant (upper part), and the pellet (lower part). After centrifugation, the supernatant and pellet samples were extracted with a mixture of pentane

and diethyl ether in a 3:1 ratio. The samples were prepared in duplicates and stored in vials at -20 °C until further analysis.

The stability of the target compound was calculated according to Eq. 2:

$$\text{Stability (\%)} = (\text{sample}_{\text{concentration after digestion}} / \text{sample}_{\text{concentration before digestion}}) \times 100\% \quad (2)$$

Gas chromatography-tandem mass spectrometry (GC-MS/MS)

The benzyl isothiocyanate concentration was determined by a gas chromatograph model 8890 GC coupled to the tandem mass spectrometer model 7000D GC/TQ (GC-MS/MS) equipped with an automatic liquid injector model 7693A (Agilent Inc., Santa Clara, CA, USA). A non-polar HP-5MS UI column (dimensions: 30 m long, 0.25 mm inner diameter and 0.25 stationary phase layer thickness, Agilent Inc., Santa Clara, CA, USA) was used. The temperature program was as follows: the initial temperature 60 °C, set isothermally for 3 minutes, and then heated to 246 °C at a rate of 10 °C min^{-1}, and maintained for 3 min isothermally. The other chromatographic conditions were as follows: The carrier gas was helium; the flow rate was 1 mL/min; the inlet was heated to 250 °C, the volume of the analysed sample was 1 μL, and the split ratio was 1:50.
A tandem mass spectrometer (MS/MS) was used in MRM recording mode, and other conditions were as follows: The ionization potential was 70 eV; the temperature of the ion source was 230 °C; the auxiliary heater temperature was 280 °C, the temperature of the first and second quadrupole (Q1 and Q2) was 150 °C, while the flow of nitrogen through the collision cell was 1.5, the recording delay due to the solvent was 2.1 minutes.
During the quantitative determination of benzyl isothiocyanate (BITC), the following characteristic transitions (fragments) were monitored with precisely defined collision energies, in order to obtain the highest possible response:

149 → 91 m/z @ 8 eV quantifying;
149 → 65 m/z @ 40 eV qualifying;
91 → 39 m/z @ 36 eV qualifying;
91 → 65 m/z @ 18 eV qualifying.

Using this method, a calibration curve with a concentration range of 0.01 to 1.0 mg/mL (y = 28090340.79x - 95140.46; R2 = 0.9979) was obtained.

Results and discussion

Microwave-assisted distillation (MAD) is a simple method which includes placing fresh plant material in a microwave reactor without adding any solvent or water. The internal heating of the *in-situ* water within the fresh plant material stretches the plant cells and leads to a rupture of the glands and oil vessels, releasing essential oil (Mohamadi et al., 2013).
The yield of the essential oil obtained from the seeds of the *T. majus* L. *altum* (TMA) by MAD was 0.06%. According to Blažević et al. (2020b), the yield of essential oil obtained from plants that contain glucosinolates using MAD was lower than the yield obtained using conventional techniques. Despite the lower yield, MAD offers other advantages like shorter extraction times, less energy consumption, and a reduced environmental hazard (less CO_2 ejected in the atmosphere) (Moradi et al., 2018).
The GC-MS analysis showed that the essential oil contained two volatile compounds originated from the degradation of glucotropaeolin: 2-phenylacetonitrile at a retention time of 17.50 min with a relative content of 0.80%, and BITC at a retention time of 27.11 min with a relative content of 97.81% (Fig. 3).
Due to various biological activities, the content of isothiocyanates, that remains after the digestion and is available for absorption, is extremely important. The results in Table 1 represent the stability of BITC in the samples of the essential oil of TMA seeds after *in vitro* and *ex vivo* gastric and intestinal phases of digestion. The data presented in Table 2 contains the stability of the pure BITC compound after the same two-phase simulated digestion methods. The quantification of the target compound was determined by the GC-MS/MS technique, using a calibration curve with a concentration range of 0.01 to 1.0 mg/mL.
The analysis showed that the BITC from the essential oil was more stable after *in vitro* and *ex vivo* gastric digestion phases. After the *in vitro* gastric phase, the concentration of BITC in the essential oil was 97.57%, and after the *ex vivo* method of digestion it was 73.47%, when compared to the undigested sample (100%). On the other hand, when subjected to the intestinal phase of digestion, the BITC from the essential oil showed to be less stable, i.e., its concentration decreased by almost a third of its value after the *in vitro* digestion method (71.17%), and almost a half after the *ex vivo* method of digestion (54.90%). Overall, the stability rates of the BITC from the essential oil after *in vitro* (gastric and intestinal) and *ex vivo* (gastric and intestinal) phases were

69.44% and 40.34%, respectively. Thus, its stability in the stomach is significantly higher than in the intestinal phase.

After *in vitro* and *ex vivo* gastric phases, the concentrations of the pure BITC compound decreased to 79.23%, and 72.24%, respectively. The concentrations of pure BITC after *in vitro* and *ex vivo* intestinal phases decreased even more, i.e., the stability was 70.36%, and 64.70%, respectively. It can be observed that the pure BITC compound is more stable in both phases of the *in vitro* digestion method than after the *ex vivo* intestinal phase, which is in accordance with the data obtained from the experiment with BITC from the TMA essential oil. Overall, the stability rates of pure BITC after the *in vitro* (gastric and intestinal) and the *ex vivo* (gastric and intestinal) phase were 55.75%, and 46.74%, respectively.

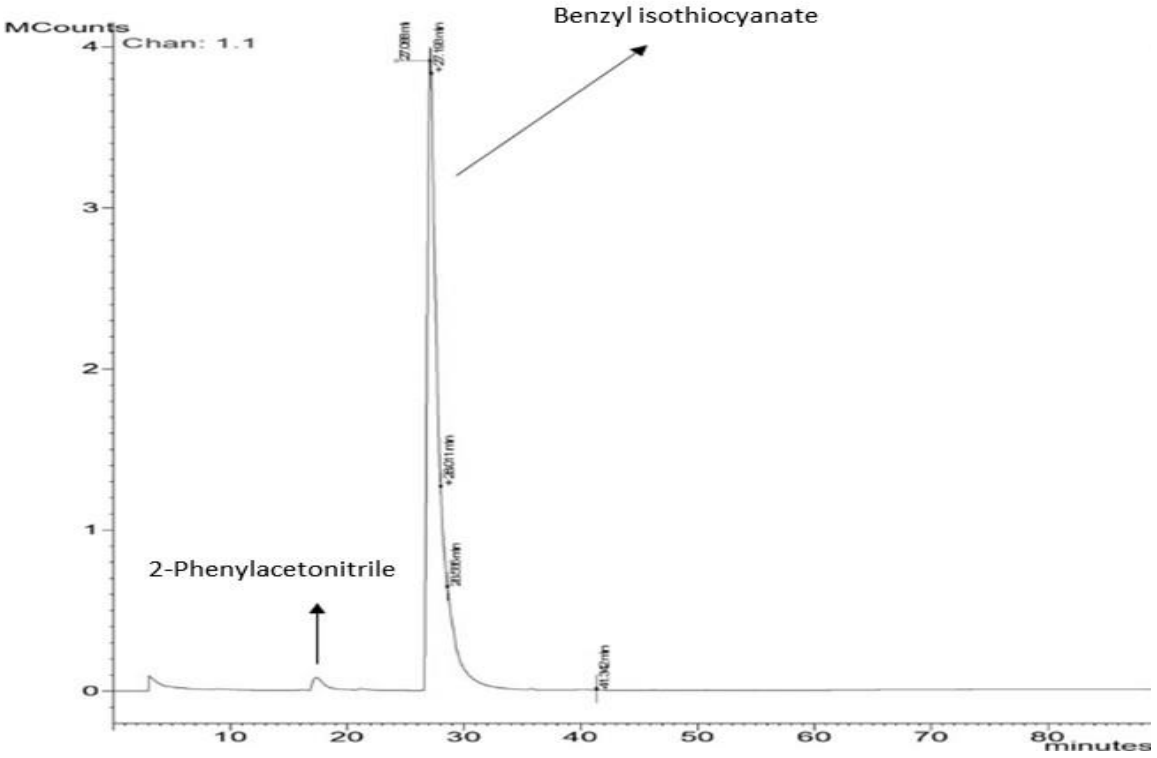

Fig. 3. Chromatogram of the volatile compounds in essential oil of the *Tropaeolum majus* L. *altum* seeds

Table 1. Stability of benzyl isothiocyanate (BITC) from *Tropaeolum majus* L. *altum* essential oil after *in vitro* and *ex vivo* digestion methods

	Concentration (mg/ml)	**Stability (%)**	**Overall stability (%)***
after gastric phase (*in vitro*)	1.62	97.57	69.44
after intestinal phase (*in vitro*)	1.19	71.17	
after gastric phase (*ex vivo*)	1.22	73.47	40.34
after intestinal phase (*ex vivo*)	0.91	54.90	

*Overall stability was calculated by multiplying percentages of gastric and intestinal phases.

Table 2. Stability of pure benzyl isothiocyanate (BITC) after *in vitro* and *ex vivo* digestion methods

	Concentration (mg/ml)	**Stability (%)**	**Overall stability (%)***
after gastric phase (*in vitro*)	1.41	79.23	55.75
after intestinal phase (*in vitro*)	1.25	70.36	
after gastric phase (*ex vivo*)	1.29	72.24	46.74
after intestinal phase (*ex vivo*)	1.15	64.70	

*Overall stability was calculated by multiplying percentages of gastric and intestinal phases

The obtained results clearly showed the differences in stability rates of BITC in pure form and incorporated in essential oil, as well as between the *in vitro* and *ex vivo* digestion methods.

In the introduction part, we already pointed out the enzymatic complexity of human digestive juices in relation to commercial digestive enzymes. The following components have been detected in human gastric juices: pepsin, trypsin, gastricsin, bile, small peptides, and protein fragments (Foltz et al., 2015). Duodenal juice contains pancreatic and intestinal enzymes such as proteolytic enzymes, intestinal lipases, enterokinases, trypsinogen, chymtrypsinogen, and amylase (Hur et al., 2011). Lower gastrointestinal stability of pure BITC in comparison with the stability of BITC within essential oil could be related to the protective effect of the matrix under gastrointestinal conditions.

It can also be assumed that one of the reasons for the different stability rate between the BITC from essential oil of TMA seeds and the pure BITC compound is that the essential oil from TMA seeds, except for BITC, contains other compounds (such as 2-phenylacetonitrile) that can contribute to the observed differences in the applied conditions, which should be further investigated. Generally, the gastrointestinal stability of isothiocyanates is poorly investigated so far (Oliviero et al., 2018) and there is no data on the gastrointestinal stability of benzyl isothiocyanate, as far as the authors know.

Conclusions

The results obtained in this study show the presence of 2-phenylacetonitrile as a minor component and benzyl isothiocyanate as a main component in the essential oil of *T. majus* L. *altum* seeds, after microwave-assisted distillation (MAD). This study showed that benzyl isothiocyanate that was obtained in the essential oil as the major compound from *T. majus* L. *altum* and its pure form show greater stability in simulated *in vitro* and *ex vivo* gastric digestion phases when compared to the intestinal phases. Also, the gastrointestinal stability of pure benzyl isothiocyanate was lower for both methods, in comparison with its stability within essential oil. Generally, the gastrointestinal stability was lower for the *ex vivo* digestion method, in comparison with the *in vitro* method.

Studies on the degree of stability of isothiocyanates are important due to their strong biological activity and thus should be further expanded with the use of optimal encapsulate formulations, in order to preserve their stability during digestion and their availability in the human body.

Ethical confirmation: The study included an *ex vivo* digestion method with human digestive juices. Permission for the collection of human digestive juices was obtained by the Ethics Committee of the Clinical Hospital Center - Split and the Ethics Committee of the Faculty of Medicine, University of Zagreb, Reg. No.: 380-59-10106-20-111/105, Class: 641-01/20-02/01.

Author Contributions: Chemistry investigation: I.V. and I.B.; obtaining human gastric juices: A.B., two-phase digestion simulations: I.V. and T.B.; tandem mass spectrometry analysis: F.B.; writing–original draft: I.V. and T.B.; writing–review & editing: all authors. All authors have read and agreed to the published version of the manuscript.

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