

# Development of an *in vitro* model to simulate the gastrointestinal digestion and absorption of stabilizing agents

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**Abstract.** Each step in the winemaking process must be carefully planned and controlled to optimize the quality of wine. Among others, tartaric stabilization is a critical step in enology, and although effective, the usual practices to solve it show some qualitative limitations, and important economical and environmental impacts. For the reasons reported above, the wine producers are searching for alternative practices, with particular interest in the area of organic products. Biopolymers are possible alternatives in this field. The selection, the characterization and the safety aspects of new biopolymers are the objectives of the European project STABIWINE (Use of biopolymers for sustainable stabilization of quality wines). The first group of biopolymers analyzed includes polyaminoacids and, in particular, polymers of L-aspartic acid (PAA), which can be used as enological additives for tartaric stabilization. In order to contribute in drafting the toxicological dossier, the metabolic fate of PAAs has been assessed by *in vitro* models, mimicking gastrointestinal digestion and absorption.

## 1. Introduction

The modern consumer likes a wine with bright and clear aspect, so clarification and stabilization are important parts of the winemaking process. Clarification is accomplished by racking, fining, filtration and aging. Stabilization is obtained by adding to the previous treatments chilling, ion exchange, and the use of special additives.

One frequent cause of wine instability is the formation of potassium bitartrate crystals, which is more evident at low temperatures, as a consequence of a decreased solubility. Metatartaric acid and proton exchangers are often used for the stabilisation of wines, as well as cold treatments; among them, the most widespread treatment consists in maintaining the wine for one week at a temperature close to freezing.

Although effective, the usual practices for wine stabilization show some qualitative limitations, and important economical and environmental impacts. At present, the practices for tartaric, protein and oxidative stabilization are not suitable for each type of wine and production facility; for example, significant problems have been observed in small wineries (conventional and organic). Moreover, they involve a large use of energy or water, can negatively affect overall wine quality and processing, require the use of large quantities of coadjuvants or additives, and can be at the origine of significant product losses.

A possible solution is offered by the use of biopolymers; these additives are suitable candidates to provide new flexible, selective and inexpensive tools for wine stabilization, which are more respectful of wine quality and environment, and well tuned with the requests of modern wine consumers.

The identification and the study of the most suitable biopolymers for winemaking are the objects of the European project named STABIWINE (“Use of biopolymers for sustainable stabilization of quality wines”, developed within the European Union’s Seventh Framework Programme). The research is focused on polyaminoacids, and in particular on polyaspartates (PAAs), new anti-scaling additives, with an exponential diffusion in other sectors thanks to their positive combination of efficacy, safety and price. The polyaspartates are obtained by polymerization – through simple heating – of aspartic acid, a widespread aminoacid; the compounds obtained have very high surface charge, and peculiar physical and chemical characteristics, which contribute to their suitability for winemaking practices.

The study was aimed to produce first data useful to the safety assessment of PAAs; in particular, the main objectives were 1) to evaluate the proteolysis of PAAs, using an *in vitro* model, which simulates the gastrointestinal digestion, and 2) to estimate the intestinal absorption by using CaCo-2 cells.

## 2. Materials and method

To simulate the human gastrointestinal digestion of polyaspartates, an *in vitro* model was developed, optimizing a sequential proteolytic attack by pepsin (from porcine gastric mucosa, 0,7 FIP-U/mg, EC 3.3.23.1, Merck, Darmstadt, Germany), and pancreatin (from porcine pancreas, 350 FIP-U/mg protease, 6000 FIP-U/mg lipase, 7500 FIP-U/mg amylase, EC 232.468.9, Merck, Darmstadt). Seven commercial PAAs, with different chemical characteristics, were included in the study; they are listed in Table 1.

**Table 1.** Products included in the experimental activity.

Code of product	Form	PAA content (%)	Average MW (kDa)	Cation
PAA-A	Powder	80	3	Na
PAA-B	Liquid	30.5	3	Na
PAA-C	Liquid	28.6	10	K
PAA-D	Liquid	31.2	10	Na
PAA-E	Liquid	31.2	15	Na
PAA-F	Liquid	29.2	5	K
PAA-G	Powder	90.6	5	K

## 2.1. *In vitro* sequential gastrointestinal proteolysis

### 2.1.1. *In vitro* gastric digestion

Different amounts of PAAs (equivalent to 24 mg of polyaminoacid) were suspended in 8 mL of 0.06 N HCl (pH 1-2) containing 0.05 mg/mL of pepsin. The final protein concentration was 3 mg/mL and the enzyme/protein ratio was 1:60 (w/w). Final pH value ranged between 1.27 and 2.80, where pepsin shows the strongest activity.

The samples were incubated at 37 °C for 5 min, 10 min or 2 h, in a Dubnoff water bath shaken at 100 beats/min. Samples from gastric digestion were treated as described below and analysed for proteolysis, or further digested by pancreatin.

All digestions were performed in duplicate. At each selected time of hydrolysis, samples were heated at 100 °C for 10 min to stop the enzymatic activity. A “peptic blank” sample was obtained by heating (without incubation) all reagents, including enzymes, for 10 min at 100 °C.

### 2.1.2. *In vitro* intestinal digestion

At the end of gastric digestion (2 h of peptic attack), borate buffer (2M boric acid, 1 N NaOH) containing 0.5 mg/mL pancreatin was added in the ratio 1:3.5 (v/v) to the solution. The hydrolysis lasted 4 or 24 hours.

All digestions were performed in duplicate. At each selected time of hydrolysis, samples were heated at 100 °C for 10 min to stop the enzymatic activity. A “pancreatic blank” sample was obtained as described for peptic digestion.

## 2.2. Quantification of proteolysis

Proteolysis was monitored by quantifying: a) the undigested proteins by microbiuret method; and b) the release of aminoacids by ninhydrin method.

### 2.2.1. Microbiuret assay

Microbiuret assay is used to quantify proteins thanks to a specific reaction between peptidic bonds and an alkaline reagent containing copper sulfate. The absorbance measured at 310 nm (UV-visible spectrophotometer, Varian Cary 50 SCAN, Palo Alto, California, U.S.A.) is directly proportional to the concentration of polypeptides, representing the residual proteins (undigested material).

The polypeptides were measured in the supernatant from different digestion times, according to Itzhaki and Gill [1]. PAA-G was used as a standard to obtain the linear regressions (one for gastric, and another for intestinal samples).

After different times of digestion, two aliquots of supernatants were added with 1) copper sulfate solution 0,2% in sodium hydroxide, or 2) sodium hydroxide (30% in water). The absorbance at 310 nm of the second aliquot was subtracted from that of copper reagent solution.

The polypeptide concentrations in digested samples were determined by using a linear regression where  $\Delta$  310 nm absorbances ( $A_{\text{copper sulfate}} - A_{\text{sodium hydroxide}}$ ) were associated with known PAA concentrations.

### 2.2.2. Ninhydrin assay

Ninhydrin is commonly used for the detection of primary amines, including aminoacids, which react with this reagent forming an intense purple derivative. In this study, the absorbance measured at 570 nm (UV-visible spectrophotometer, Varian Cary 50 SCAN, Palo Alto, California, USA) is proportional to the quantity of amino acid released during digestion [2,3].

At selected times of digestion, aliquots of supernatant were added with 2% ninhydrin solution. All solutions were mixed, heated for 15 minutes at 100 °C, and finally cool down in ice for 10 minutes.

Two linear regressions were prepared with standard L-aspartic acid: 1) in 0.06 N HCl for peptic digestion, and 2) in HCl/borate buffer in the ratio 1:3.5 (v/v) for pancreatic attack.

## 2.3. *In vitro* assays with CaCo-2 cells to simulate intestinal absorption of PAA

The effect of the selected compound on the integrity of the gastrointestinal barrier was assessed using differentiated CaCo-2 cells as a model of intestinal barrier. [4]. Cytotoxicity was as assessed by MTT reduction; while the barrier integrity by the trans-epithelial electrical resistance (TEER), using 20% of ethanol as a positive control. Inflammation was assessed by measuring the release of IL-6, IL-8.

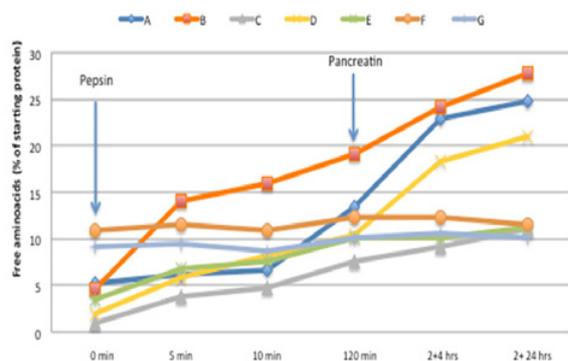
In parallel, the absorption through intestinal barrier was measured by microbiuret assay, in apical and basolateral portion of CaCo-2 cells after 24 hours treatment at 37 °C.

In this assay, biopolymer PAA-G was digested as described above; T0 (undigested) and T2h (pepsin)+T24 h (pancreatin) were added to CaCo-2 cells.

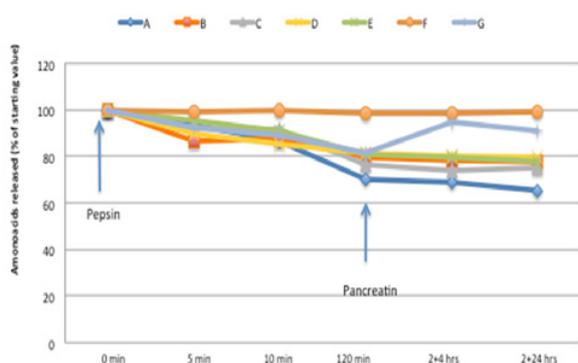
## 3. Results

### 3.1. Quantification of proteolysis

To assess the metabolic fate of polyaminoacids, a method suitable to quantify the rate of digestion was necessary. Unfortunately, the chemical nature of the polyaspartate is not suitable for the methods usually applied in protein quantification. In fact, most of them [5,6] are based on the reaction between aromatic aminoacids (present in protein sequence) and the colorimetric reagents.



**Figure 1.** Aminoacid release during *in vitro* gastrointestinal digestion of PAAs. Data indicate the progression of proteolysis, expressed as the percentage of aminoacids released/total protein.



**Figure 2.** Undigested PAAs during *in vitro* gastrointestinal digestion. Data indicate the progression of proteolysis, expressed as the percentage of residual polypeptides/total protein.

Due to the lack of aromatic rings, these methods are unsuitable for this research and proteolysis was measured applying the microbiuret method as described in 2.2.1; this test measures the peptidic bonds in polypeptides and can be used to quantify the undigested PAAs. The release of aminoacids (as a measure of progressive hydrolysis) was quantified by the ninhydrin method (2.2.2).

The proteolytic profiles of the PAA samples are shown in Fig. 1 (free aminoacids) and Fig. 2 (undigested PAA). The proteolytic patterns obtained in the *in vitro* gastrointestinal model show that all PAAs are very poorly digested, with minimal pancreatic attack. In fact, the values of released aminoacids range between 10 and 30% after 24 h of pancreatic attack, while those of residual polypeptides are always above 60%. Table 2 shows data of PAA-G, the most promising product.

The values reported as “free aminoacids” could be partially overestimated, since also terminal amino groups of PAAs can react with ninhydrin.

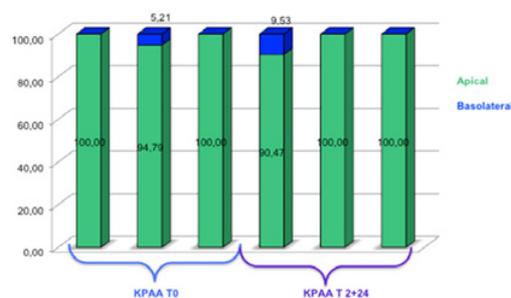
### 3.2. Intestinal permeability

In the preliminar enological tests, PAA-G was selected as the most promising product, so it was included in the intestinal integrity/permeability study.

PAA-G was added to differentiated CaCo-2 cells as a model of intestinal barrier. No changes in barrier integrity

**Table 2.** Proteolytic profile of PAA-G, as assayed in the optimized *in vitro* gastrointestinal digestion model.

Enzyme	Digestion time	Undigested protein (% of starting value)	Free aminoacids (% of total protein)
Pepsin	0 min	100	10.90 ± 0.02
Pepsin	5 min	99.36 ± 4.38	11.48 ± 0.01
Pepsin	10 min	99.95 ± 10.91	10.95 ± 0.01
Pepsin	120 min	98.43 ± 8.20	12.30 ± 0.01
Pepsin + Pancreatin	2+4 hrs	98.48 ± 13.65	12.26 ± 0.04
Pepsin + Pancreatin	2+24 hrs	99.36 ± 9.93	11.48 ± 0.02



**Figure 3.** PAA in the apical and basolateral compartments after CaCo-2 treatment with T0 and T2+24 samples. Data are expressed as percentage of polypeptide distribution.

or in cytokine release were observed, indicating that the compound is well tolerated (data not shown).

Figure 3 shows the PAA distribution in the apical and basolateral portion of cells, after 24 hours treatment at 37 °C. Undigested PAA (T0) and sample from the last time of proteolysis (T2+24 h) were included in the assay.

The presence of polypeptides in the apical area ranges between 98.3% and 96.8% for undigested and T2+24 h sample, respectively.

The amount of PAAs dosed in basolateral portion is very limited in both samples. The insignificant/very limited absorption of PAA through the intestinal barrier, confirms data obtained by *in vitro* proteolysis. In other words, PAAs and, in particular PAA-G, are poorly digested and absorbed.

## 4. Conclusions

The proteolytic profile obtained with the optimized gastrointestinal model shows a low digestibility of PAAs; the highest level of attack is obtained on PAA-B, where aminoacid release reached 28% of total protein.

The proteolysis is practically limited to the pepsin activity. The pancreatic hydrolysis seems ineffective and this is due to the specific composition of PAA containing only acid aminoacids. It is well known that endopeptidases (trypsin and chymotrypsin), contained in

pancreatic juice, are capable to attack only peptidic bounds including basic aminoacids. The weak proteolysis observed during pancreatic attack is probably due to exopeptidases (carboxy- and aminopeptidases).

The distribution of PAA-G, evaluated in the apical and basolateral portion of CaCo-2 cells with microbiuret assay, suggests that there is a minimal absorption through the cell monolayer, since after 24 hrs from addition most PAA was still found in the apical area of CaCo-2 cells scarcely absorbed.

Data obtained in this study are the first step for the preparation of toxicological dossier. Low digestion and absorption are positive aspects in term of safety.

These results together with those obtained in laboratory animals will contribute to define the toxicological profile of the selected PAAs, as new wine additives.

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