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8 4 **Transepithelial transport of dry-cured ham peptides with ACE**  
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10 5 **inhibitory activity through a Caco-2 cell monolayer**  
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1  
2 30 **Abstract**

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4 31 ~~Angiotensin converting enzyme (ACE) inhibitory peptides are been extensively studied~~  
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6 32 ~~as an alternative to synthetic drugs for the treatment of hypertension. Recent studies~~  
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8 33 ~~have shown that dry-cured ham is an important source of naturally generated bioactive~~  
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10 34 ~~peptides, especially showing ACE inhibitory activity. However, due to their excessive~~  
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12 35 ~~degradation by digestive and brush border enzymes, it is not clear whether these~~  
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14 36 ~~peptides resist intestinal absorption and reach the blood stream where they may exert~~  
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16 37 ~~their antihypertensive effect. So, d~~Dry-cured ham extracts and specific pure peptides  
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18 38 naturally generated during the dry-curing process, showing ACE inhibitory activity,  
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20 39 have been studied for their stability during transepithelial transport in a Caco-2 cell  
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22 40 monolayer. The ACE inhibitory activity of transport samples was assayed, reaching the  
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24 41 highest values in apical samples after 15 min ~~of~~ incubation. In basal solutions, the  
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26 42 highest ACE inhibition was observed for peptides AAPLAP and KPVAAP after 60 min  
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28 43 of cellular transport. However, when basal samples were four times concentrated, a  
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30 44 considerable increased ACE inhibitory activity was observed ~~in these peptides from 15~~  
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32 45 ~~min of incubation~~. Fragments generated by cellular activity were detected by using  
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34 46 tandem mass spectrometry MS techniques, showing that AAATP, AAPLAP, and  
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36 47 KPVAAP were hydrolysed during the transport, although KPVAAP was also absorbed  
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38 48 intact~~ly~~. This study highlights the potential of intact dry-cured ham peptides as well as  
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40 49 their fragments to be absorbed across the intestinal epithelium and reach the blood  
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42 50 stream to exert an antihypertensive action.

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50 53 *Keywords:* Dry-cured ham, ACE inhibitory peptides, Caco-2 cell monolayer, intestinal  
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52 54 transport, mass spectrometry.

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2 56 **1. Introduction**

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4 57 Angiotensin I converting enzyme (ACE) is a dipeptidyl carboxypeptidase which plays  
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6 58 an essential role as a regulator of blood pressure. ACE converts angiotensin I to the  
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8 59 potent vasoconstrictor angiotensin II, which also induces the release of aldosterone.  
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10 60 Moreover, ACE inactivates bradykinin, which has vasodilator activity. As a result, the  
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12 61 action of ACE on these two systems is responsible for hypertension, the most common  
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14 62 type of cardiovascular disease (Skeggs, Kahn, & Shumway, 1956; Ondetti, Rubin, &  
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16 63 Cushman, 1977; Unger, 2002). To exert effects on blood pressure, ACE-inhibitory  
17  
18 64 compounds ~~such as antihypertensive peptides~~ need to resist the degradation by  
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20 65 gastrointestinal proteases and brush border peptidases, be absorbed through the  
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22 66 intestinal epithelium, and finally reach the bloodstream in an active form (Vermeirssen,  
23  
24 67 Augustijns, Morel, Van Camp, Opsomer, & Verstraete, 2005).

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26  
27 68 Spanish dry-cured ham has recently been investigated as a natural source of  
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29 69 antihypertensive peptides, evaluating the ACE inhibitory activity of water soluble  
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31 70 fractions of dry-cured ham extracts (Escudero, Aristoy, Nishimura, Arihara, & Toldrá,  
32  
33 71 2012), and identifying some of the peptides responsible for this inhibitory effect  
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35 72 (Escudero, Mora, Fraser, Aristoy, Arihara, & Toldrá, 2013). Moreover, Escudero, Mora,  
36  
37 73 and Toldrá (2014) have recently reported that the ACE inhibitory activity of dry-cured  
38  
39 74 ham peptides persists after *in vitro* digestion with gastric proteases, which may be due  
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41 75 to ~~both resistance~~ the stability of the existing antihypertensive peptides to digestion ~~as~~  
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43 76 ~~well as~~ and the generation of small fragments ~~with~~ showing ACE inhibitory activity.  
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46 77 Furthermore, the *in vivo* antihypertensive activity of dry-cured ham extracts and peptide  
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48 78 AAATP has been studied, showing a decrease in systolic blood pressure after their oral  
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50 79 administration to spontaneously hypertensive rats (SHR) (Escudero et al., 2012;  
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52 80 Escudero et al., 2013).

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2 81 Transport assays through Caco-2 cells, which is a cell line derived from human colon  
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4 82 adenocarcinoma, have been established as a model for small intestinal transport of drugs  
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6 83 and food compounds. Differentiated Caco-2 cells maintain the morphology and function  
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8 84 of mature enterocytes and express brush border ~~proteases-peptidases and transporters~~  
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10 85 ~~and peptidases~~ that may affect peptide stability and transport, being therefore utilised to  
11  
12 86 predict the absorption in the small intestine (Hidalgo, Raub, & Borchardt, 1989; Yee,  
13  
14 87 1997). In this ~~senseregard~~, several studies have been focused on studying the  
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16 88 transepithelial transport of antihypertensive peptides derived from different food  
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18 89 products such as egg or milk in a qualitative way (Miguel, Dávalos, Manso, De la Peña,  
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20 90 Lasunción, & López-Fandiño, 2004; Vermeirssen et al., 2005; Quirós, Dávalos,  
21  
22 91 Lasunción, Ramos, & Recio, 2008; Bejjani & Wu, 2013). However, to the best of our  
23  
24 92 knowledge, there are no transport studies based on peptides showing ACE inhibitory  
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26 93 activity derived from meat or meat products. In this work, the Caco-2 cell line was used  
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28 94 to study the brush border degradation and transepithelial transport of ACE inhibitory  
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30 95 peptides derived from dry-cured ham. In contrast with other studies focused on the  
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32 96 ACE-inhibitory activity of food peptides, this study investigates the ACE-inhibitory  
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34 97 effect of dry-cured ham peptides transported through the intestinal epithelium and hence  
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36 98 show their potential to exert an antihypertensive action *in vivo*.  
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38 99 ~~In addition, the ACE inhibitory activity of the transported peptides was measured to~~  
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40 100 ~~evaluate their final antihypertensive potential.~~  
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## 102 2. Materials and methods

### 103 2.1 Material and reagents

104 Dulbecco's Modified Eagle's Medium (DMEM), GlutaMAX™, phosphate buffered  
105 saline (PBS), and nonessential amino acids were procured from Life Technologies

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2 106 (Ghent, Belgium), whereas fetal bovine serum was from Greiner Bio-One (Vilvoorde,  
3  
4 107 Belgium). Angiotensin-converting enzyme (from rabbit lung) was purchased from  
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6 108 Sigma Chemical Co. (St. Louis, Mo., USA), and [o-aminobenzoylglycyl-p-nitro-L-](#)  
7  
8 109 [phenylalanyl-L-proline](#) (Abz-Gly-p-nitro-Phe-Pro-OH) trifluoroacetate salt was from  
9  
10 110 Bachem AG. (Bubendorf, Switzerland). [Methanol HPLC grade was from Sharlab, S.L.](#)  
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12 111 [\(Barcelona, Spain\)](#). All other chemicals and reagents used were of analytical grade.  
13

## 14 112 **2.2 Dry-cured ham extracts and peptides**

15  
16 113 The study was done using extracts from different types of dry-cured ham. Samples M1  
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18 114 and S1 were obtained from Spanish dry-cured hams with ten months of processing,  
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20 115 which were ~~submitted~~subjected to extraction and deproteinisation according to the  
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22 116 methodology described by [Escudero et al. \(2012\)](#). In the case of sample S1, the extract  
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24 117 was fractionated by gel filtration chromatography [using a Sephadex G-25 column \(2.5 ×](#)  
25  
26 118 [65 cm\)](#), and fractions corresponding to elution volumes from 200 to 320 mL were  
27  
28 119 pooled together and ~~lyophilised~~dried. These fractions were selected because they have  
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30 120 shown the maximum ACE inhibitory activity, reaching values of 80% of inhibition in a  
31  
32 121 previous study ([Escudero et al., 2012](#)). In addition, Designation of Origin of Teruel  
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34 122 hams with a minimum time of ripening of fourteen months were used to obtain samples  
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36 123 M2 and M3. Peptide extraction was done according to the method described by  
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38 124 [Escudero et al. \(2014\)](#) for sample M2, while sample M3 was ~~submitted~~subjected to  
39  
40 125 extraction and deproteinisation following the same procedure as described above for  
41  
42 126 sample M1 and S1. All dry-cured ham extracts were desalted by solid phase extraction  
43  
44 127 using an Oasis<sup>®</sup> [hydrophilic-lipophilic balance \(HLB\)](#) cartridge (35 cc, Waters, Ireland),  
45  
46 128 where peptides were retained and then eluted with 50% methanol. Finally, the eluates  
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48 129 were lyophilised for the transport experiment across Caco-2 cells.  
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2 130 Since dry-cured ham extract samples contain a complex mixture of peptides that  
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4 131 difficult a detailed study, three peptides (AAATP, AAPLAP, and KPVAAP) previously  
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6 132 identified as ACE inhibitors in dry-cured ham (Escudero et al., 2013; Escudero et al.,  
7  
8 133 2014) were selected. In fact, peptides AAPLAP and KPVAAP were chosen based on  
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10 134 their potent ACE inhibition, with IC<sub>50</sub> values of 14.38 and 12.37 μM, respectively.  
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12 135 Whereas, AAATP (IC<sub>50</sub> value of 100 μM) was selected for its good *in vivo*  
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14 136 antihypertensive action in the SHR model (Escudero et al., 2013). These three peptides  
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16 137 were synthesised by GenScript Corporation (Piscataway, NJ, USA) at the highest purity  
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18 138 certified using liquid chromatography - mass spectrometry (LC-MS) ~~LC-MS~~ analysis  
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20 139 for subsequent transport experiments.

## 22 140 **2.2 Caco-2 cell culture**

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24  
25 141 Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD,  
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27 142 USA). Cells were grown in DMEM containing 4.5 g/L glucose, supplemented with  
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29 143 GlutaMAX™, 10% fetal bovine serum and 1% nonessential amino acids, at 37 °C in a  
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31 144 humidified atmosphere containing 10% CO<sub>2</sub>. The passage number of the cells used in  
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33 145 this study was between 30 and 35. For transport experiments, cells were seeded at a  
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35 146 density of 20,000 cells/well on high throughput screening (HTS) Transwell®-24 well  
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37 147 permeable supports (0.4 μm pore polyester membrane, 6.5 mm inserts, 0.33 cm<sup>2</sup> cell  
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39 148 growth area; Costar, Corning, Birmingham, UK). The culture medium was replaced  
40  
41 149 every 2-3 days and cells were allowed to differentiate for at least 21 days before  
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43 150 experiments. Cell monolayer integrity was checked visually by phase-contrast  
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45 151 microscopy, and the transepithelial electrical resistance (TEER) of Caco-2 cells of this  
46  
47 152 batch assessed with an automated tissue resistance measurement system (REMS, World  
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49 153 Precision Instruments, Hertfordshire, UK) was higher than 300 Ω\*cm<sup>2</sup>.

## 52 154 **2.3 Transport studies**

1  
2 155 Differentiated Caco-2 cells were gently rinsed twice with PBS and then incubated with  
3  
4 156 PBS for 1 day at 37 °C in 10% CO<sub>2</sub> prior to the transport assays. After removing the  
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6 157 PBS from all wells, 1 mg/mL of the samples dissolved in PBS ~~were was~~ added to the  
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8 158 apical chambers (200 µL), whereas fresh PBS was added to the basolateral chambers (1  
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10 159 mL). A control sample, containing only PBS and no peptides, was included in the  
11  
12 160 experimental setup, and samples from apical and basolateral sides were taken before (0  
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14 161 min) and during incubation at different time points of 15, 30, and 60 min. The action of  
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16 162 cell proteases on samples after the transport study was immediately stopped by adding 9  
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18 163 volumes of methanol, centrifuged at 7500g for 10 min, and finally the supernatant  
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20 164 containing the peptides was taken and dried for the following analysis. All experiments  
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22 165 were ~~conducted done~~ in triplicate.

#### 25 166 **2.4 ACE inhibitory activity**

26  
27 167 Apical and basal samples taken from the transepithelial transport at different times were  
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29 168 analysed for ACE inhibitory activity. For this purpose, dried apical samples were  
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31 169 dissolved to the original volume in bidistilled water, whereas dried basal samples were  
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33 170 first dissolved in a volume four-fold smaller than the original one to determine the ACE  
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35 171 inhibition in concentrated samples, and then ~~redissolved diluted~~ to the original volume  
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37 172 to test again. Basal samples were concentrated in order to be comparable in terms of  
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39 173 peptide concentration with apical samples. Additionally, the ACE inhibitory activity of  
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41 174 several synthesised fragments derived from the degradation of AAATP, AAPLAP, and  
42  
43 175 KPVAAP through the cellular transport was evaluated. ~~In all studied cases, t~~The ACE  
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45 176 inhibitory activity was measured according to the method developed by [Sentandreu and](#)  
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47 177 [Toldrá \(2006\)](#), which is based on the ability of ACE to hydrolyse the internally  
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49 178 quenched fluorescent substrate ~~o-aminobenzoyl-glycyl-p-nitro-L-phenylalanyl-L-proline~~  
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51 179 (~~Abz-Gly-Phe(NO<sub>2</sub>)-Pro~~). The assay was done in triplicate. ACE inhibition of samples  
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180 is ~~expressed~~showed as ACE inhibitory percentage and ~~as~~IC<sub>50</sub> value, which is the  
181 peptide concentration that inhibits 50% of ACE activity in the reaction mixture.

## 182 **2.5 MALDI-ToF/ToF mass spectrometry**

183 Apical and basal samples taken after the transport of peptides AAATP, AAPLAP, and  
184 KPVAAP across Caco-2 cells were analysed using matrix-assisted laser  
185 desorption/ionisation time-of-flight/time-of-flight (MALDI-ToF/ToF) mass  
186 spectrometry to determine the molecular mass of the peptide mixture and detect those  
187 possible peptides resulting from the degradation of the precursor peptides. The analysis  
188 was done in MS mode, and a total of 1  $\mu$ L of every sample was directly spotted on the  
189 MALDI plate and allowed to air dry. Then, 0.5  $\mu$ L of matrix solution (which contains 5  
190 mg/mL of  $\alpha$ -cyano-4-hydroxycinnamic acid (HCCA; Bruker Daltonics, Germany) in  
191 0.1% ~~TFA-ACN/H<sub>2</sub>O~~trifluoroacetic acid - acetonitrile/water (7:3, v/v) was spotted. The  
192 analysis was done in a 5800 MALDI ToF/ToF instrument (AB Sciex, MA, USA) in  
193 positive reflectron mode (3000 shots every position) in the range from 150 to 800 Da  
194 m/z range; the laser intensity was manually adjusted to maximize the S/N ratio. The  
195 system was adjusted with voltages of 15 and 3 kV in the source and reflector detector,  
196 respectively. Previously, the plate model and the acquisition methods were calibrated by  
197 AB Sciex calibration mixture (1 fmol/ $\mu$ L des-Arg1-Bradykinin; 2 fmol/ $\mu$ L Angiotensin  
198 I; 1.3 fmol/ $\mu$ L Glu1-Fibrinopeptide B; 2 fmol/ $\mu$ L Adrenocorticotropic hormone  
199 (ACTH) (1–17 clip); 5 fmol/ $\mu$ L ACTH (18–39 clip); and 3 fmol/ $\mu$ L ACTH (7–38 clip))  
200 in 13 positions.

201 The candidate precursors were then selected for every position in the MS/MS  
202 (ToF/ToF) analysis in order to confirm their presence. In this senseregard, ten of the  
203 most intense precursors according to the threshold criteria minimum signal-to-noise: 10,  
204 minimum cluster area: 500, maximum precursor gap: 200 ppm, and maximum fraction



1  
2 205 gap: 4, were selected. The MS/MS method was calibrated using Angiotensin I MS/MS  
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4 206 spectra in 13 positions, and data was acquired using the default 1kV MS/MS method.  
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6 207 Manual analysis of data was done using mMass – Open Source Mass Spectrometry Tool  
7  
8 208 Software v.5.5 (<http://www.mmass.org>) (Strohalm, Kavan, Novák, Volný, & Havlíček,  
9  
10 209 2010). The search of peptide sequences previously identified showing ACE inhibitory  
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12 210 activity was done using BIOPEP database  
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14 211 (<http://www.uwm.edu.pl/biochemia/index.php/en/biopep>).  
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## 16 212 **2.6 Statistical analysis**

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18 213 Statistical analysis was done using Statgraphics Centurion XVI software. One-way  
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20 214 analysis of variance (ANOVA) was performed and ~~the~~ mean and standard deviation  
21  
22 215 were reported. Fisher's multiple range test was carried out to analyse significant  
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24 216 differences among mean values at  $p < 0.05$ .  
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## 28 218 **3. Results and discussion**

### 29 219 **3.1 Transepithelial transport of peptides across Caco-2 cell monolayer**

30  
31 220 In order to simplify the study of dry-cured ham extracts which contain a complex  
32  
33 221 mixture of thousands of peptides, the apical to basal flux transport in Caco-2 cell  
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35 222 monolayers was mainly studied with the purified peptides (AAATP, AAPLAP, and  
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37 223 KPVAAP). For that, 1 mg/mL of every sample was added to the apical side, and  
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39 224 samples were taken from both apical and basal compartments at different times: 0, 15,  
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41 225 30, and 60 min.  
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45 227 The evolution of peptides AAATP, AAPLAP, and KPVAAP during their transcellular  
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47 228 transport was then monitored by using MALDI-ToF mass spectrometry to determine the  
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49 229 profile of peptides according to their molecular masses. As illustrated in [Figure 1](#), there  
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1 230 the apical ones, suggesting a partial degradation of the precursor peptides into smaller  
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4 231 fragments by the action of cell peptidases ~~as well as~~ and their absorption through the  
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6 232 Caco-2 cell monolayer. The size, amino acid sequence, charge, hydrogen-bonding  
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8 233 capacity, non-enzymatic glycosylation, and hydrophobicity of peptides are critical in  
9  
10 234 determining their susceptibility to peptidases and their permeability across the intestinal  
11  
12 235 epithelium (Pauletti, Okumu, & Borchardt, 1997; Shimizu, Tsunogai, & Arai, 1997;  
13  
14 236 Artursson, Palm, & Luthman, 2001).

### 16 237 **3.2 ACE inhibitory activity of samples taken from transport assays**

18 238 Dry-cured ham extracts and peptide solutions taken from the apical and basal  
19  
20 239 compartments at different transport times were analysed for ACE inhibitory activity ~~5~~  
21  
22 240 ~~directly after sampling and in basal samples also after concentrating four times~~ (Figure  
23  
24 241 2). In the case of dry-cured ham extracts, s Samples taken from the apical side showed  
25  
26 242 high ACE inhibition, ~~generally~~ reaching the maximum values at 15 min of cellular  
27  
28 243 transport except for sample S1, which presented slightly higher ACE inhibition before  
29  
30 244 incubation than during the cellular assay. in both types of samples, and with values very  
31  
32 245 close to 100% inhibition for the purified peptides. These high inhibition values at 15  
33  
34 246 min of the transport may be due to a strong action of cell peptidases on these peptides ~~at~~  
35  
36 247 ~~this time~~, whereby the modification of the peptide structure may affect its biological  
37  
38 248 activity and peptide fragments released can show even a higher degree of ACE  
39  
40 249 inhibitory activity ~~compared to~~ in comparison with the precursor peptides (Miguel,  
41  
42 250 Recio, Gómez-Ruiz, Ramos, & López-Fandiño, 2004). In fact, In the case of dry-cured  
43  
44 251 ham samples, samples M1 and S1 showed similar inhibition results even though sample  
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46 252 S1 contained only the fractions which showing the highest ACE inhibitory activity.  
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48 253 Meanwhile, the activity decreases more significantly after 30 min of transport for  
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50 254 samples M2 and M3, which come from dry-cured hams with longer curing process. The  
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2 255 ACE inhibition activity for sample M2 before the transport assay was very low (with a  
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4 256 value of 4.5%), which is in agreement with a previous study (Escudero et al., 2014); and  
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6 257 ~~which that~~ is probably ~~due owing~~ to this sample has not been deproteinised ~~compared~~  
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8 258 ~~to in comparison with~~ sample M3. Additionally, sample M2 did not show any ACE  
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10 259 inhibitory activity in the apical side after 60 min of cellular transport, indicating that  
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12 260 inhibitory peptides were either completely hydrolysed or transported to the basal side.  
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14 261 ~~On the other hand, little to no ACE inhibition was detected in the basal samples, so For~~  
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16 262 ~~this reason, all basal samples they~~ were concentrated four times to check if samples may  
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18 263 show ACE inhibitory activity at higher concentration. Under these conditions, dry-cured  
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20 264 ham extract samples showed activity although to a varying degree, obtaining values  
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22 265 around 30-40% at 15 min of transport and then decreasing during time.  
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24 266 ~~With regard to purified peptides, Figure 2 evidences ACE inhibition values very close~~  
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26 267 ~~to 100% in apical samples at 15 min of the transport. On the other hand, little to no~~  
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28 268 ~~ACE inhibition was detected in the basal samples, except significant results were only~~  
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30 269 ~~found~~ for peptides AAPLAP and KPVAAP after 60 min of transport, whereby ACE  
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32 270 inhibitory activity reached values of 42% and 30%, respectively. However, ~~for purified~~  
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34 271 ~~peptides in the case of concentrated basal samples,~~ the ACE inhibitory activity of  
35  
36 272 peptides AAATP and AAPLAP increased during incubation, reaching values up to 30%  
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38 273 for AAATP and 70% for AAPLAP at 60 min of the assay. ~~Also~~ high percentages of  
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40 274 inhibition (around 65%) were also observed for KPVAAP after 15 and 60 min of  
41  
42 275 cellular transport.  
43  
44 276 These results indicate that some peptides ~~as well as~~ along with small derived fragments  
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46 277 could be absorbed, maintaining their ACE inhibitory activity after cellular transport  
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48 278 although at low concentration in non-concentrated basal samples (Figure 2). Moreover,  
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50 279 synergic effects between antihypertensive peptides could explain higher values of ACE  
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2 280 | inhibition for peptide mixtures in comparison to the individual activity of ~~individual~~  
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4 281 | peptides. In addition, it should be ~~stressed-highlighted~~ that the concentration of peptides  
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6 282 | in the basal and concentrated basal samples does not show a linear but exponential  
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8 283 | response when ACE activity is plotted against the concentration of ~~an~~-ACE inhibitory  
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10 284 | peptides~~s~~, as previously described by [Sentandreu and Toldrà, \(2006\)](#).  
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12 285 | Because angiotensin I converting enzyme is present in the brush border contributing to  
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14 286 | hydrolysis of peptides ([Yoshioka, Erickson, Woodley, Gulli, Guan, & Kim, 1987](#)),  
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16 287 | control samples containing only the Caco-2 cells and PBS were also analysed for ACE  
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18 288 | inhibitory activity, showing non-significant effects (data not shown). Nevertheless,  
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20 289 | these values were taken into account for determining the percentage of ACE inhibition  
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22 290 | of the samples.

### 25 291 **3.3 Peptide degradation by epithelial peptidases**

26  
27 292 | As dry-cured ham samples contain a very complex mixture of peptides, only the  
28  
29 293 | degradation of the purified peptides on the brush-border side was evaluated by MS-  
30  
31 294 | based techniques in order to detect those peptides that were transcellularly transported.  
32  
33 295 | [Table 1](#) shows the peptide sequences detected by MALDI-ToF/ToF MS in the apical  
34  
35 296 | and basal compartments at the different assayed times.

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37 297 | The analysis revealed the degradation of the intact peptide AAATP after 15 min of  
38  
39 298 | transport ~~with-and the~~ generation of smaller sized peptides, ~~some-of-which~~ ~~can-could~~  
40  
41 299 | cross the intestinal barrier up to the basal side. On the other hand, AAPLAP was  
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43 300 | detected in the apical chamber throughout the experiment, but analysis of the basal  
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45 301 | solutions showed that it was not transported intact. In fact, several shorter fragments  
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47 302 | were also detected in the apical and basal solutions, suggesting its partial degradation by  
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49 303 | brush border peptidases. Furthermore, peptide KPVAAP was detected up to 60 min of  
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51 304 | transport in both apical and basal samples, indicating that its chemical and structural  
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1 305 properties allow it to be absorbed intact through the Caco-2 monolayer. However, it was  
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3  
4 306 also susceptible to peptidases and different derived fragments were also detected in  
5  
6 307 apical and basal solutions. In this ~~senseregard~~, among all the fragments detected in  
7  
8 308 either apical or basal samples, several of them were previously reported as ACE  
9  
10 309 inhibitory peptides ([Table 1 of Supplementary material2](#)), ~~being LAP and VAA those~~  
11  
12 310 ~~with the lowest IC<sub>50</sub> values~~. The remaining peptides detected at different times of  
13  
14 311 incubation as shown in [Table 1](#) (AATP, ATP, AAAT, APLA, PLAP, AAPL, VAAP,  
15  
16 312 KPV, AAA, and VA) were synthesised and assayed for the *in vitro* ACE inhibitory  
17  
18 313 activity in order to calculate their IC<sub>50</sub> [values](#) ([Table 32](#)). These results highlight the  
19  
20 314 potent inhibition of fragment VAAP resulting from KPVAAP degradation, and PLAP  
21  
22 315 from AAPLAP, which showed IC<sub>50</sub> values of 16.75 and 76.5 μM, respectively. [This fact](#)  
23  
24 316 [could be explained by the presence of hydrophobic amino acids such as proline and](#)  
25  
26 317 [alanine close to the C-terminal position of the peptides that positively influence the](#)  
27  
28 318 [binding to ACE \(Rohrbach, Williams, & Rolstad, 1981; Majumder, & Wu, 2009\).](#)  
29  
30 319 Hence, the ACE inhibitory activity shown by the peptide fragments could also explain  
31  
32 320 the inhibitory activity found previously in apical and basal samples (see [Figure 2-b](#)),  
33  
34 321 suggesting that those fragments transported across the monolayer could also reach the  
35  
36 322 blood stream to exert an antihypertensive activity. As such, brush border peptidases  
37  
38 323 play a key role in the formation and degradation of bioactive peptides and therefore, in  
39  
40 324 their bioavailability and physiological effect ([Pihlanto-Leppälä, 2000; Vermeirssen, et](#)  
41  
42 325 [al., 2005; Miguel & Aleixandre, 2006](#)).  
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44  
45 326 The study done by [Escudero et al. \(2013\)](#) showed *in vivo* ACE inhibitory activity for the  
46  
47 327 peptide AAATP, although according to the present study it is not absorbed intact  
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49 328 through the intestinal barrier ([Table 1](#)). These results suggest that either the fragments  
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51 329 derived from its degradation are responsible for the decrease in blood pressure in the  
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1 330 SHR model, or AAATP may exert antihypertensive activity through the interaction with  
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4 331 receptors expressed in the gastrointestinal epithelia (Yamada, Matoba, Usiu, Onishi, &  
5  
6 332 Yoshikawa, 2002), thereby inducing other mechanisms of blood pressure regulation  
7  
8 333 such as ~~through~~ nitric oxide or endothelin production (Lifton, Gharavi, & Geller, et al.,  
9  
10 334 2001).

11  
12 335 The stability of peptides to enzymatic hydrolysis and absorption processes determine  
13  
14 336 their bioavailability and bioactivity, being the main cause of differences found between  
15  
16 337 *in vitro* and *in vivo* assays (Pihlanto-Leppälä, 2000; Vermeirssen, Van Camp, &  
17  
18 338 Verstraete, 2004; Bejjani, & Wu, 2013). In fact, the amount of peptides absorbed *in*  
19  
20 339 *vivo* could be higher than *in vitro* assays when Caco-2 cells are used as models, ~~due~~  
21  
22 340 owing to the lower expression of some intestinal transporters and tighter junctions in  
23  
24 341 Caco-2 cell monolayers ~~compared to~~ in comparison with *in vivo* intestinal tissues  
25  
26 342 (Lennernäs, Palm, Fagerholm, & Artursson, 1996; Boisset, Botham, Haegele, Lenfant,  
27  
28 343 & Pachot, 2000; Vermeirssen et al., 2005). Nevertheless, this study suggests a beneficial  
29  
30 344 impact of dry-cured ham peptides towards blood pressure due to their potential to exert  
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32 345 an *in vivo* antihypertensive action.  
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#### 37 347 **4. Conclusions**

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39 348 Previous studies reported the ACE inhibitory activity of some dry-cured ham peptides  
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41 349 and their stability to *in vitro* digestion. However, antihypertensive peptides need to  
42  
43 350 resist the complete hydrolysis by brush-border peptidases and be absorbed actively  
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45 351 across the intestinal epithelium to exert their activity. The transepithelial transport  
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47 352 through a Caco-2 cell monolayer has been studied in dry-cured ham extracts and  
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49 353 purified peptides, evaluating the degradation of peptides by mass spectrometry. Results  
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51 354 showed that peptides AAATP, AAPLAP, and KPVAAP are degraded throughout the  
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1  
2 355 transport assay, although KPVAAP can also be absorbed intact through the intestinal  
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4 356 barrier. Moreover, this study evidences for the first time the absorption and generation  
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6 357 of ACE inhibitory peptide fragments originating from dry-cured ham by the Caco-2 cell  
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8 358 line. The antihypertensive action of the peptides or small fragments derived as well as  
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10 359 the synergic effect between them could explain the ACE inhibitory results obtained in  
11  
12 360 apical and basal samples, suggesting that dry-cured ham peptides could reach the  
13  
14 361 circulatory system to exert an antihypertensive action.  
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16 362

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21 482 **FIGURE CAPTIONS**

22  
23 483 **Figure 1.** MALDI-ToF mass spectra of samples taken from the transepithelial transport  
24  
25 484 (apical and basal sides) through Caco-2 cell monolayer at different times (0, 15, 30, 60  
26  
27 485 min) after adding the purified peptides a) AAATP, b) AAPLAP, and c) KPVAAP.

28  
29 486 **Figure 2.** ACE inhibitory activity (%) observed in the apical, basal, and four times  
30  
31 487 concentrated basal compartments obtained from the cellular transport after the  
32  
33 488 application of a) dry-cured ham extracts and b) purified peptides. ~~DifferentBar~~ letters ~~in~~  
34  
35 489 indicate significant differences at  $p < 0.05$  (n=3) between different times of the cellular  
36  
37 490 transport (0, 15, 30, 60 min) in each compartment and sample ( a = apical; a = basal 4x;  
38  
39 491 a = basal).  
40  
41 492 ~~sample and compartment indicate significant differences at  $p < 0.05$  (n=3).~~

**Table 1.** Peptide sequences detected by using MALDI-ToF/ToF MS in the cellular transport assay.

Precursor peptide	Peptide fragments <sup>a</sup>	Monoisotopic mass (Da) <sup>b</sup>	Apical – times (min) <sup>c</sup>				Basal – times (r	
			0	15	30	60	15	30
AAATP*		429.22	x	x				
	AATP	358.19		x	x	x		
	AAAT	332.17						x
	ATP	287.15		x			x	x
	AAA	231.12						x
AAPLAP*		538.31	x	x	x	x		
	PLAP	396.24		x	x	x		
	APLA	370.44					x	x
	AAPL	370.22					x	x
	PL *	228.15					x	x
	LA *	202.13						
KPVAAP*		581.35	x	x	x	x	x	x
	VAAP	356.21			x		x	x
	KPV	342.23			x		x	x
	KP*	243.16		x		x		
	VA	188.12		x	x	x	x	x
	AP*	186.10		x	x	x		

\*Sequences previously identified showing ACE inhibitory activity according to BIOPEP database (see [1]).

<sup>a</sup> Possible fragments derived from the degradation of the precursor peptide.

<sup>b</sup> Monoisotopic molecular mass in Daltons of the matched peptide.

<sup>c</sup> Peptides detected in the apical compartment at different transport times.

<sup>d</sup> Peptides detected in the basal compartment at different transport times.

**Table 2.** ACE inhibitory activity ( $IC_{50}$ ) of synthetic peptides.

Peptides	$IC_{50}$ ( $\mu$ M)
VAAP	16.75
PLAP	76.50
AAA	111.47
AATP	300.74
ATP	406.56
AAAT	513.65
VA	607.96
KPV	> 1000
AAPL	> 1000
APLA	> 1000

Figure 1

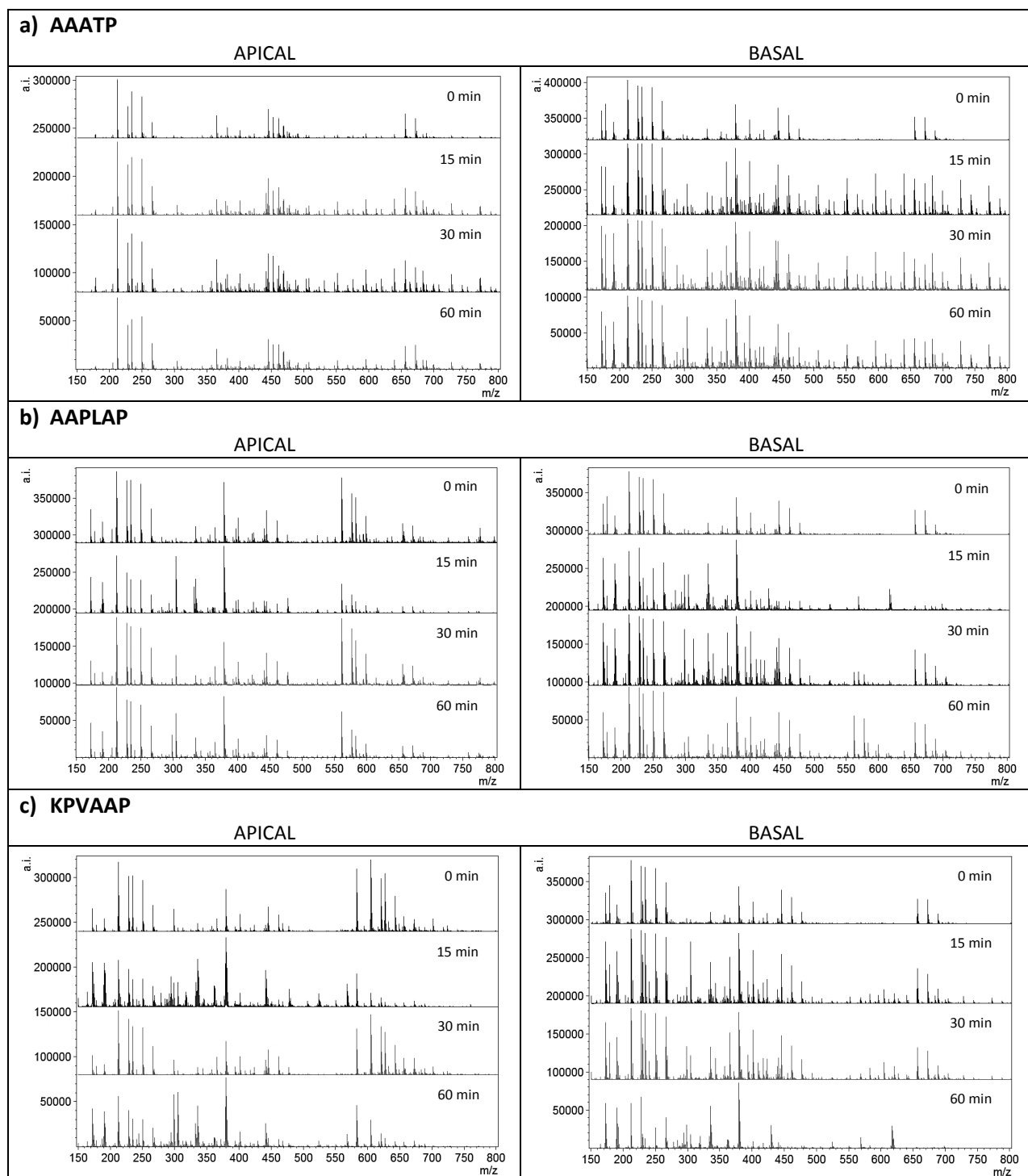


Figure 1.

Figure 2

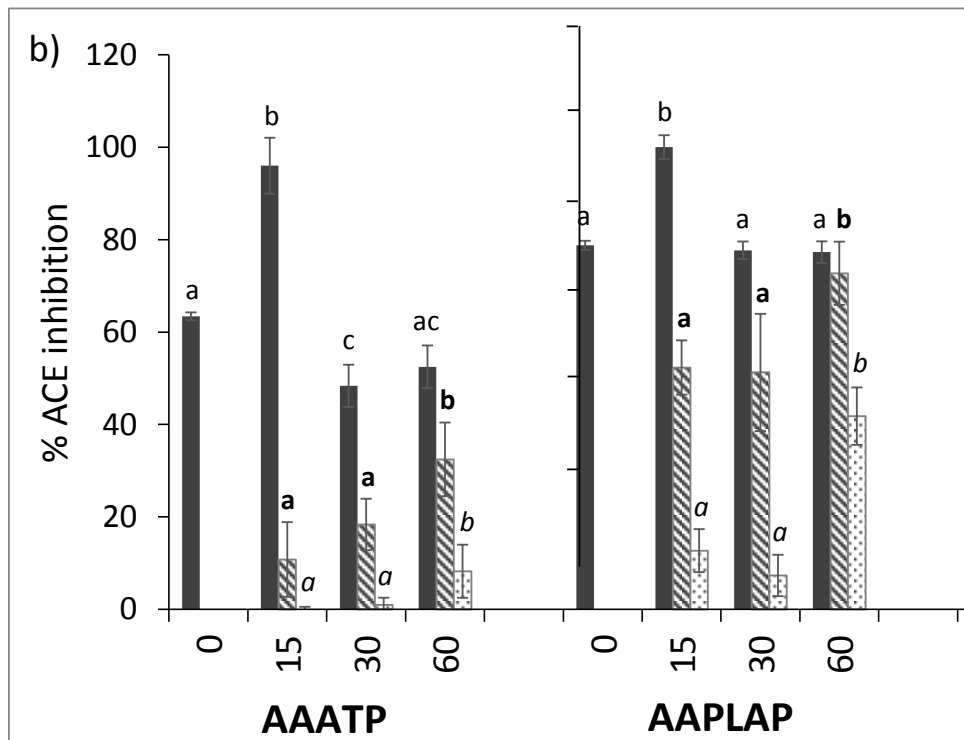
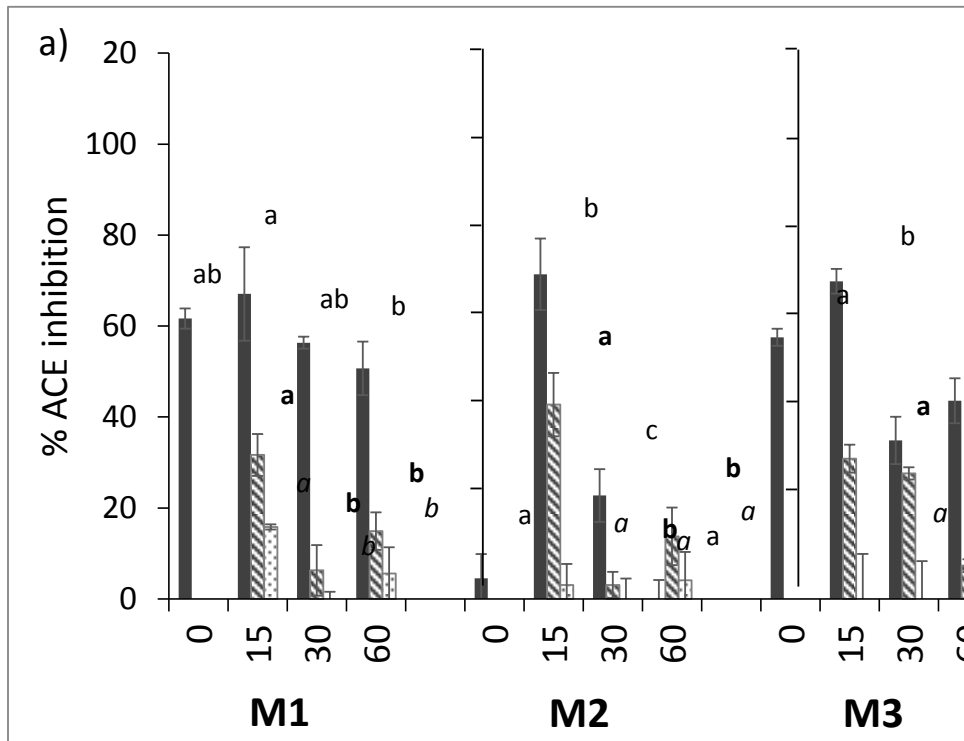


Figure 2.



**Supplementary Table 1.** Pep

Peptides	IC <sub>50</sub> (μM)
KPVAAP	12.37
AAPLAP	14.38
KP	22
AAATP	100
AP	230
LA	310
PL	337.32