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Improved liquid-chromatography tandem mass spectrometry method for the determination of the bioactive dipeptides, carnosine and anserine: Application to analysis in chicken broth

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ABSTRACT

An improved method, based on ultra-performance liquid chromatography (UPLC) coupled to tandem mass spectrometry (MS/MS), has been developed to determine the bioactive dipeptides carnosine (CAR) and anserine (ANS) in chicken broth. These analytes are hydrophilic (polar) and in order to improve their retention, the chromatographic mode used was hydrophilic interaction chromatography (HILIC) (1.7 μm particle size). In order to remove the salt before the chromatographic analysis of the chicken broth (0.8%, w/w), an exhaustive sample pre-treatment strategy was necessary since the salt is not volatilized and could block the ionization source and lead to signal suppression. The chicken broth was firstly centrifuged to remove the fat and chicken proteins, and then was pretreated by off-line solid-phase extraction (SPE), using traditional cartridges, or off-line μElution plate (μSPE), using microplates, and the results were compared. Due to the high polar character of the dipeptides studied and the sample matrix, these compounds were not retained in the sorbent hydrophilic–lipophilic balanced (HLB) and were eluted in the load step, whereas the salt was retained in the sorbent. This fact was observed by the addition of silver nitrate in the chicken broth extract, where before the SPE or μSPE a white precipitate (silver chloride) was formed and after the SPE or μSPE this precipitate was not observed. By using these sample pre-treatment strategies, the extraction recoveries were higher than 80%, and the matrix effect was lower than 12%. Once the improved method was developed, the quality parameters of the method were studied. The LODs and LOQs of the CAR and ANS were lower than 6 and 1.8 μg/l, respectively. Then, the method was applied to analyse a commercial chicken broth. This improved method allowed determining CAR and ANS between 6 and 10 mg dipeptide/l chicken broth in 10 min (sample pre-treatment and chromatographic analysis). Therefore, the proposed improved method is concluded to be rapid, sensitive and selective for the determination of polar compounds by MS in samples that contain salt.

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1. Introduction

Many dietary proteins contain different peptide sequences encrypted within their primary structure that exert beneficial effects upon human health once released by digestive enzymes during gastrointestinal transit or by food processing [1,2]. The low molecular weight peptide, carnosine (β-alanyl-L-histidine, CAR) is the archetype of a class of aminoacyl histidine dipeptides that includes homocarnosine (γ-amino-butyryl-histidine, HCAR), anserine (*N*-β-alanyl-3-methyl-l-histidine, ANS), and balenine (*N*-β-alanyl-1-methyl-histidine) [3]. The most important activity of these dipeptides is their ability to act as antioxidants [4,5], and these have been shown to be particularly effective in preventing damage and/or the death of neurons caused by exposure to oxidative stress [6–12]. Recently, Boldyrev et al. [13] showed that the histidine-containing dipeptide family, with CAR often showing the highest effectiveness, positively influenced several aging-related molecular processes in cerebral tissue during neurodegenerative disorders (Alzheimer, Parkinson, etc.).

In humans, the diet is the main source of CAR and its derivatives, which are found in significant amounts in red and white meats (beef, chicken and pork) and fish [14]. Chicken broth is an excellent

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food: it is a low-calorie food that is part of the Mediterranean diet and that helps to maintain health [15] and manage weight [16]. Furthermore, starting a meal with broth prepares the gut for digestion. In fact the consumption of chicken broth has increased in the recent years (to 16.4% of Spanish homes in 2010) as a result of the consumer not having time to cook but wanting to eat healthily. Additionally, chicken broth is a good source of water, which helps to maintain hydration, and of such micronutrients as dipeptides, CAR and ANS, with biological benefits (bioactive dipeptides) [17].

The bioactive peptides CAR and ANS have been analysed both in meat [14,18,19] and feed [20,21] samples, and in biological samples [3,22–24]. The analytical separation techniques most useful for analysing meat and feed samples are liquid-chromatography (LC) using the reserved-phase [14,19], ion-exchange [20,21], and hydrophilic interaction chromatography modes (HILIC) [18]. Regarding to the detection system, photodiode array (PDA) detection with or without post-column derivatization [14,18,20], amperometric detection [21] and mass spectrometry (MS) [19] have been reported for the analysis of these bioactive dipeptides. These methodologies are based on conventional LC, where the stationary phases have a particle size between 3 μm [18,19] and 5 μm [14,20,21], and an inner diameter of 4.6 mm. Recently, ultra-performance LC (UPLC) has been developed as a result of the improvement in the packing materials used for chromatographic separations. This technique takes the advantage of chromatographic principles to run separations using columns packed with smaller size particles (such as 1.7 μm) and smaller inner diameter (2.1 mm). As a result, speed is increased and the resolution and sensitivity is higher than in the conventional LC [25,26].

As well as the analytical separation technique, meat broths have to be pretreated before chromatographic analysis to remove interference compounds, such as salt, especially if the detector system is MS, since salt leads to signal suppression. In addition, the salt is not volatilized and could block the ionization source. To date, to the best of our knowledge, there are no studies of the chromatographic analysis of meat broths in the literature. The use of a micellar phase as the mobile phase has been reported for the analysis of CAR and ANS in meat by direct injection without long and tedious extraction procedures [14]. Nevertheless, this mobile phase is not compatible with the MS as the detection system, since the micellar phase is not volatilized. However, different strategies have been reported for desalting samples, such as an improved sample clean-up method, based on dialysis and solid-phase extraction (SPE), or/and an efficient chromatographic separations with reversed-phase HPLC and size-exclusion chromatography [27]. Other authors used micropipette SPE for desalting protein samples, but these products are costly, and require numerous manual handling steps, and, in addition, are only useful for analysing biomolecules, such as proteins [28,29].

The aim of this study was to develop an improved method, in terms of speed, sensitivity and selectivity, in order to determine the bioactive dipeptides CAR and ANS in chicken broth. The improved analytical method proposed is based on UPLC, by using the HILIC mode, coupled to tandem MS. As a strategy for chicken broth pretreatment, centrifuging and both off-line SPE and off-line μElution plates (μSPE) were used to remove the interferences (such as salt), and the results were then compared. To the best of our knowledge, this is the first report in which these bioactive dipeptides have been analysed by UPLC and by using tandem MS as the detection system, and also the first time these analytes have been analysed in chicken broth.

2. Experimental

2.1. Chemicals and reagents

Standards of CAR and ANS were purchased from Sigma (St. Louis, MO, USA). Stock solutions of individual standards were prepared by dissolving each compound in acetonitrile/Milli-Q water (75/25, v/v) at a concentration of 1000 mg/l and stored in a dark flask at 4 °C. A standard stock mix of the standards was prepared weekly at a concentration of 50 mg/l. Table 1 shows the chemical structure of CAR and ANS.

Methanol (HPLC grade), acetonitrile (HPLC grade), acetic acid were all provided by Scharlau Chemie (Sentmenat, Barcelona, Spain). Water was Milli-Q quality (Millipore Corp, Bedford, MA, USA). Ammonium acetate was from Sigma (St. Louis, MO, USA), and silver nitrate was purchased from Acros Organics (Geel, Belgium).

2.2. Samples

Commercial chicken broth was supplied by Gallina Blanca Star with the following composition: water, chicken (0.8%), vegetables in variable proportions (0.04%) (such as onion, celery, leeks, carrots and potatoes), lard, salt (0.8%), yeast extract, flavoring, and

Table 1
Chemical structure and SRM conditions used for UPLC–MS/MS determination of the bioactive dipeptides studied. The quantification transition is shown in bold.

Dipeptide	Chemical structure	Log P (o-w)	Molecular weight (g/mol)	Ionization mode	Transitions	Cone voltage (V)	Collision energy (eV)
CAR	O, NH₂, NH, NH, N, OH, O	−1.81[a]	226	Positive	**227.1 > 109.8**	30	25
					227.1 > 109.8	30	15
ANS	O, NH₂, NH, N, N, OH, O, CH₃	−1.26[a]	240	Positive	**241.1 > 109**	30	20
					241.1 > 170.3	30	15

[a] log *P* (octanol-water) [31].

spices. The nutritional information per 100 ml of chicken broth was: energy (20 KJ; 5 Kcal), protein (0.3 g), fat (0.4 g) and sodium (0.3 g).

2.3. Broth sample pre-treatment strategy

Chicken broth was pre-treated before the chromatographic analysis in order to clean-up the sample matrix by removing the interference compounds and salt. 10 ml of chicken broth were centrifuged at 8163 rpm for 20 min at 4 °C. The obtained supernatant was filtered through glass wool (Scharlau, Sentmenat, Barcelona), and then through a 0.22 μm nylon filter (Whatman International Ltd. Maidstone, England). Afterwards, the filtered sample was diluted (10-fold or 100-fold) with 0.2% acetic acid and loaded into SPE cartridges or μSPE plates, in order to compare the results. The 100-fold dilution was used to study the quality parameters of the method, and the 10-fold and 100-fold dilutions were used to apply the method, and the obtained results with the two dilutions tested were compared.

The SPE cartridges used were OASIS HLB 60 mg (Waters Corp., Milford, MA). These cartridges were conditioned by adding sequentially 5 ml of methanol and 5 ml of 0.2% acetic acid (pH 3.5). Then, 1 ml of diluted chicken broth sample was loaded into the cartridge, and the eluted sample was collected. On the other hand, the μSPE plates used were OASIS hydrophilic–lipophilic balanced (HLB) packed with 2 mg of sorbent (Waters Corp., Milford, MA). These plates were conditioned by adding sequentially 250 μl of methanol and 250 μl of 0.2% acetic acid (pH 3.5). Then, 350 μl of diluted chicken broth and 350 μl of 0.2% acetic acid were loaded into the plate, and the eluted sample was collected. The collected sample was then diluted 2-fold with acetonitrile/Milli-Q water (75/25, v/v) solution, filtered through a 0.22 μm nylon filter and injected into the chromatographic system.

The presence of salt (sodium chloride) before and after the sample pre-treatment strategy was evaluated by adding silver nitrate. If a white precipitate (silver chloride) is observed, it means the sample contains salt.

2.4. UPLC–MS/MS

The UPLC analysis of CAR and ANS in the pre-treated broth sample was performed using a Waters Acquity™ UPLC system (Waters, Milford, MA, USA), equipped with a Waters binary pump system (Waters, USA). The column was an AcQuity UPLC™ BEH HILIC (100 mm × 2.1 mm i.d., 1.7 μm), also from Waters. The mobile phase was 0.65 mM ammonium acetate with Milli-Q water/acetonitrile (25/75, v/v) as eluent A, and 4.55 mM ammonium acetate with Milli-Q water/acetonitrile (70/30, v/v) as eluent B. The elution started at 5% of eluent B and was increased linearly to 11% of eluent B in 6 min, further increased to 100% of eluent B in 0.1 min and kept isocratic for 1.4 min. It was then returned to the initial conditions in 0.1 min, and the reequilibration time was 1.4 min. The flow-rate was 0.4 ml/min, and the temperature of the column during the analysis was 30 °C. The injection volume was 10 μl, and all the samples were filtered through 0.22 μm nylon filters (Whatman) before the chromatographic analyses.

The UPLC system was coupled to a PDA detector Acquity UPLC™ and a TQD™ mass spectrometer (Waters, USA). The PDA detector wavelength was set at 214 nm. Ionization was achieved with an electrospray (ESI) interface operating in the positive mode $[M-H]^+$ and the data were collected in the selected reaction monitoring (SRM). The ionization source parameters were capillary voltage, 3 kV; source temperature, 120 °C; cone gas flow-rate, 5 l/h and desolvation gas flow-rate, 800 l/h; desolvation temperature 400° C. Nitrogen (99.99% purity, N_2LCMS nitrogen generator, Claind, Lenno, Italy) and argon (≥99.99% purity, Aphagaz, Madrid, Spain) were used as cone and collision gases respectively.

The SRM transitions and the individual cone voltage and collision energy for each dipeptide were evaluated by infusing 10 mg/l of each compound to obtain the best instrumental conditions. Two SRM transitions were studied to find the most abundant product ions, the most sensitive transition being selected for quantification and a second one for confirmation purposes. Table 1 shows the MS/MS transitions for quantification (in bold) and confirmation, as well as the cone voltage and the collision energy values optimized for each of the standard compounds. The dwell time established for each transition was 30 ms, and the software used was MassLynx 4.1.

2.5. Quality parameters

The instrumental quality parameters of the analytical method, such as the linearity, calibration curve, repeatability, robustness, detection limits (LODs) and quantification limits (LOQs) were determined for diluted chicken broth (100-fold) spiked with known concentrations of dipeptide compounds and extracted according to the procedure described in Section 2.3. These quality parameters were according the food and drug administration (FDA) guidelines [30].

2.6. Statistical analysis

The CAR and ANS concentrations in the analysis of chicken broth were analyzed by the ANOVA test to assess the effect of both dilution sample matrices (10-fold and 100-fold), and the sample pre-treatment used (μSPE or SPE as the device format). A significant difference is considered at a level of $P < 0.05$. All the statistical analysis was carried out using STATGRAPHICS Plus 5.1.

3. Results and discussion

3.1. HILIC as the analytical column

In the literature, different liquid chromatographic (LC) methodologies have been reported to determine CAR and ANS in meat and feed samples. These methodologies are based on the reversed-phase [14,19], ionic exchange [20,21], and HILIC [18] modes.

In our study, the initial experiments to analyse the CAR and ANS dipeptides were based on UPLC by using reversed-phase and tandem MS as the detection system. Two columns were tested, the first one being the Acquity UPLC BEH C_{18} (2.1 mm × 100 mm, 1.7 μm) and the second one the Acquity UPLC BEH300C_{18} (2.1 mm × 50 mm, 1.7 μm). As the mobile phase, 0.2% acetic acid was used as eluent A and acetonitrile as eluent B. Unfortunately, under these conditions, the dipeptides were not eluted in the chromatogram in the range from 5 to 100%B. This could be explained because these dipeptides are hydrophilic (polar) and these were not retained in the reversed-phase mode. As a consequence, these compounds were eluted in the void volume of the column. Table 1 also shows its octanol–water partition coefficient (log P, o/w), and it can be seen, these values are very low and it demonstrate its hydrophilicity [31].

In order to increase its retention efficacy in the chromatographic analysis, the HILIC chromatographic mode was proposed. This chromatographic mode has been used to improve the retention of the very polar analytes. In addition, by using this LC mode coupled to MS as the detection system, sensitivity is enhanced in comparison with the reserved-phase mode, since highly volatile mobile phases are used, and these are ideal for efficient desolvation and compound ionization [32–34].

Mora et al. [18] reported a HILIC chromatographic method to determine CAR and ANS by conventional LC (HPLC) and with UV as the detector system. Therefore, in our study, an improved methodology was proposed to analyse these dipeptides by using the HILIC mode through UPLC coupled to tandem MS. The stationary phase

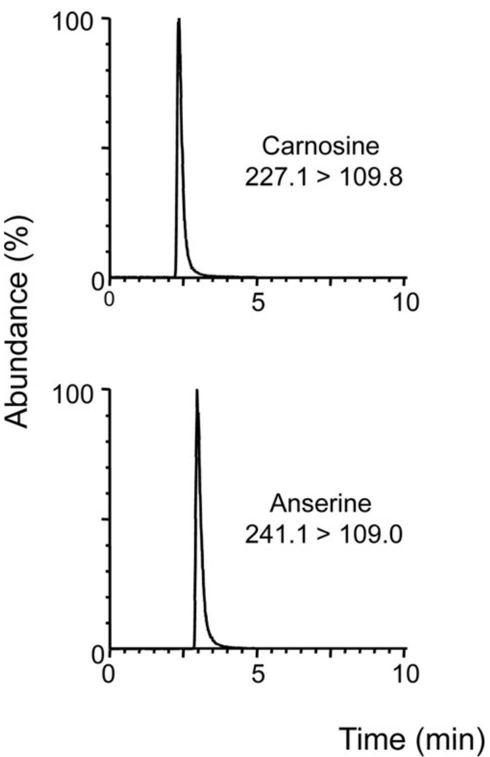

Fig. 1. Extracted ion chromatograms obtained for the analysis of CAR and ANS in standard solutions by using the chromatographic mode HILIC. The concentration of these bioactive peptides was 1 mg/l for CAR and 1.5 mg/l for ANS.

was packed with 1.7 μm size particles, and the resolution, sensitivity and speed were improved as a result [25,26].

The first experiments were based on the method reported by Mora et al. [18]. Therefore, the HPLC gradient was converted to UPLC conditions, and for this, the AcQuity UPLC™ Columns calculator (Waters, Milford, USA) was used. Then, the gradient was slightly modified in order to improve the resolution of the two dipeptides. The 1.4 ml/min flow-rate was changed to 0.4 ml/min, to be the optimum in UPLC [25,26]. Fig. 1 shows the extracted ion chromatograms of CAR and ANS by UPLC–MS/MS using the HILIC mode. The concentrations of CAR and ANS were 1 mg/l and 1.5 mg/l, respectively, and the dipeptides were prepared in acetonitrile/Milli-Q water (75/25, v/v). The retention times of CAR and ANS were 2.4 min and 3.0 min, respectively. Although the dipeptides were eluted within 3 min, the analysis time was 9 min in order to clean up the column, return it to the initial conditions and equilibrate it.

3.2. Sample pre-treatment strategy

For the analysis of chicken broth, a sample pre-treatment is necessary prior to the chromatographic analysis in order to eliminate interference from the sample matrix, such as salt. This is very important, especially when the detector system is MS, since salt is not volatilized and the ionization source can thus be blocked and the salt markedly degrades the performance of MS. Therefore, it is very important to remove the salt before the chromatographic analysis.

The sample pre-treatment consisted of removing the chicken proteins by centrifuging, filtering the supernatant, and then diluting the sample by 10-fold or 100-fold with 0.2% acetic acid, because the analytes were concentrated. The 100-fold dilution was used as a blank sample to study the quality parameters of the method. On the other hand, the 10-fold and 100-fold dilutions were used to determine CAR and ANS in chicken broth, and the results were then compared.

After centrifuging and diluting the chicken broth sample, the salt was not still removed. This fact was observed by the formation of white precipitate after the addition of several droplets of 5% silver nitrate to this sample. The silver ions (from the silver nitrate) react with the chloride ions (from the salt of the diluted sample) and a white precipitate is formed. The sample was then accordingly cleaned-up in μSPE plates to remove the salt. Three different sorbent chemistries, HLB [35], mixed-mode anion exchange (MAX) [36] and weak-cation exchange (WCX) [36], were evaluated.

The difference between these sorbents is the retention mode. The MAX and WCX sorbents provide two retention modes, both reversed-phase and ion-exchange. These sorbents allow the salt and other interference compounds to be removed from the sample matrix. On the other hand, the HLB sorbent only provides one retention mode, reversed-phase, and the salt is removed. The diluted chicken broth (100-fold) spiked with a known concentration of the studied dipeptides was used to evaluate the μSPE efficiency of the three sorbents, HLB, MAX and WCX.

The responses of the dipeptides spiked in the diluted chicken broth before and after extraction were compared to determine the extraction recoveries (%*R*) in these different sorbents.

3.2.1. μSPE by using HLB sorbent

The first experiment for desalting the diluted chicken broth was based on the study reported by Gilar et al. [35]. These authors desalted cytochrome *c* trypsin digest as a peptides and proteins mix by using the HLB sorbent with a 96-well plate (5 mg) as the device format, and by HPLC-MS. We used their procedure to analyse CAR and ANS in chicken broth but with some modifications to the volumes, as we used a μSPE (2 mg) instead of a 96-well plate (5 mg) as the device format. Briefly, 200 μl of acetonitrile and 200 μl of 0.1% trifluoroacetic (TFA) were sequentially loaded to condition and equilibrate the micro-sorbent, respectively. Then, 350 μl of diluted chicken broth extract and 350 μl of 0.1% TFA were loaded as the sample matrix. To clean-up the micro-sorbent, 500 μl of 0.1% TFA and 200 μl of Milli-Q water were used sequentially. Finally, 2 μl × 50 μl of acetonitrile/Milli-Q water (75/25, v/v) were used to elute the retained dipeptides. In the clean-up steps, both the salt and the buffer were reported to be removed.

Under these experimental conditions, the extraction recoveries of CAR and ANS in this sorbent were very low, at around 1%. The low extraction recoveries obtained were produced because the dipeptides were not retained in the micro-cartridge, and as a consequence, they were lost in the load step. On the other hand, the salt was retained in the micro-cartridge. This fact was observed because no white precipitate was formed when several droplets of silver nitrate were added into the sample matrix collected after loading the sample.

Although different equilibration, sample matrix and clean-up solvents (0.1% TFA, 0.2% acetic acid, 4% phosphoric acetic and Milli-Q water) and elution solvents (acetonitrile/Milli-Q water (75/25, v/v), acetonitrile/methanol (75/25, v/v), methanol, and acetonitrile) were tested, and different sample volumes (from 50 μl to 350 μl) and elution volumes (from 50 μl to 500 μl) were studied, CAR and ANS were lost in the load step (80–90%), whereas the salt remained in the micro-sorbent.

3.2.2. SPE by using HLB sorbent

In order to increase the quantity of the sorbent, and therefore improve the retention of the CAR and ANS, the traditional SPE HLB (60 mg) cartridges were also proposed for the analysis of the studied dipeptides. CAR and ANS were analysed by SPE by using the conditions reported by Gilar et al. [35], but with some modifications to the volumes used, because in this case we used SPE

Table 2
Instrumental quality parameters of the developed method to determine CAR and ANS by *off-line* μSPE (HLB: 2 mg) or SPE *off-line* (HLB: 60 mg), and UPLC–MS/MS using diluted chicken broth (100-fold) spiked with the standard solutions at different concentrations.

	Off-line μSPE		Off-line SPE	
	CAR	ANS	CAR	ANS
$\%R_{Total}$	64	75	62	78
$\%R_{SPE\ or\ \mu SPE}$	92	82	98	83
% Matrix effect (%ME)	−9	12	−10	12
Linearity (μg/l)	7–3300	7–3300	2–1000	2–1000
Calibration curve (μg/l)	$y = 64.43x - 2118.4$	$y = 76.55x - 1965.4$	$y = 66.64x + 178.49$	$y = 66.56x + 643.07$
Determination coefficient (R^2)	0.999	0.994	0.999	0.996
%RSD, $n = 3$				
C_1 (3300 μg/l)	6.8	7.0	1.9[a]	5.6[a]
C_2 (665 μg/l)	1.7	4.1	4.0[b]	2.6[b]
C_3 (133 μg/l)	1.9	3.9	13.4[c]	11.9[c]
C_4 (27 μg/l)	4.7	5.2	4.7[d]	4.4[d]
C_5 (10 μg/l)	12.1	14.6	5.8[e]	12.5[e]
Accuracy, $n = 3$				
C_1 (1000 μg/l)	101	99	101	100
C_2 (200 μg/l)	95	95	95	95
C_3 (40 μg/l)	105	95	100	105
LOQ (μg/l)	6.0	6.0	2.0	2.0
LOD (μg/l)	1.8	1.8	0.6	0.6

[a] %RSD: 1000 μg/l.
[b] %RSD: 200 μg/l.
[c] %RSD: 40 μg/l.
[d] %RSD: 8 μg/l.
[e] %RSD: 4 μg/l.

(60 mg) instead of a 96-well plate (5 mg) as the device format. Briefly, 3 ml of acetonitrile and 3 ml of 0.1% TFA were sequentially loaded to condition and equilibrate the sorbent, respectively. Then, 1 ml of diluted chicken broth extract and 1 ml of 0.1% TFA were loaded as the sample matrix. 2 ml of 0.1% TFA and 2 ml of Milli-Q water were sequentially used to clean-up the sorbent. Finally, 5 ml of acetonitrile/Milli-Q water (75/25, v/v) was used to elute the retained dipeptides.

Under these conditions, the results were similar to those obtained with μSPE plates. CAR and ANS were not retained in the sorbent and they were eluted in the load step. Several parameters that affected the efficiency of the SPE, the same to the studied for μSPE plates, were then also studied. In this device format, the loaded sample ranged from 1 ml to 5 ml, and the loaded clean-up and elution solutions were from to 1 ml to 10 ml. The results obtained were the same, with the dipeptides being quantitatively lost in the load step (80–90%), whereas the salt was retained in the sorbent.

3.2.3. μSPE by using MAX and WCX sorbents

The second and the third experiments for desalting the diluted chicken broth were based on those reported by Waters (Milford, USA) to ion-exchange sorbents, such as MAX (anionic) and WCX (cationic). The reported procedure was as follows: 200 μl of methanol and 200 μl of Milli-Q water were sequentially loaded to condition and equilibrate the micro-sorbent, respectively. Then, 350 μl of diluted chicken broth and 350 μl of Milli-Q water were loaded as the sample matrix. 200 μl of 5% ammonium acetate was loaded as the clean-up solution. Finally, 2 μl × 50 μl of methanol was used to elute the uncharged analytes, and then 2 μl × 50 μl of 2% formic acid in methanol was used to elute the charged analytes.

Under these experimental conditions, the extraction recoveries of CAR and ANS in these ion-exchange sorbents were also very low, around 1%. The low extraction recoveries were also because the dipeptides were quantitatively lost in the load step. Although the different parameters that affected the efficiency extraction were studied, CAR and ANS were not retained in the WCX micro-cartridge, and the extraction recoveries were the same, at around 1%. Nevertheless, when only 50 μl of diluted chicken broth extract was loaded in the MAX sorbent and 500 μl of acetonitrile/Milli-Q water (75/25, v/v) was used to elute, the extraction recoveries were 34% and 47% for CAR and ANS, respectively.

In all of these experiments, HLB, WCX and MAX, the eluted sample matrix with the analytes (in the load step) was diluted 2-fold with acetonitrile/Milli-Q water (75/25, v/v) solution in order to improve the shape peak, because the peaks were doubled.

The lack of retention of CAR and ANS in the (micro)-cartridge was due to the sample matrix, since when these dipeptides were spiked in Milli-Q water as the sample matrix instead of diluted chicken broth, the dipeptides were retained, and the extraction recoveries were good. The salt and other interference in the chicken sample matrix meant that the dipeptides could not be retained in the (micro)-cartridge.

Although the dipeptides were not retained in the (micro)-cartridge while the salt was retained in the (micro)-cartridge, this fact could be proposed to extract the dipeptides CAR and ANS from the chicken broth extract without the interference salt. Therefore, the sample pre-treatment was based on follows, the diluted chicken broth extract was loaded into the (micro)-cartridge and then the eluted sample matrix solution was collected and diluted 2-fold with acetonitrile/Milli-Q water (75/25, v/v) solution. Then it was filtered through nylon filters and finally injected into the chromatographic system. By using this protocol, the extraction recoveries of CAR and ANS were from 80 to 90% with HLB sorbents (both SPE and μSPE) and WCX micro-sorbent, and from 30 to 50% with MAX micro-sorbent.

3.2.4. Matrix effect (%ME)

Apart from the extraction recovery (%R), the matrix effect (%ME) was also evaluated. This matrix effect, which is one of the main drawbacks of MS methods when ESI is used as the ionization technique, is observed by an increase or decrease in the response of an analyte in a sample matrix compared with the same analyte in an organic solvent, and this is due to co-eluting matrix components that compete for ionization capacity [37]. In this study, the matrix effect was evaluated by comparing the peak abundances obtained from diluted chicken broth extract spiked after sample

Table 3
Concentration (mg/l) ± standard deviation (s) of CAR and ANS in chicken broth. The %RSD is shown in parenthesis.

	Concentration (mg dipeptide/l chicken broth)			
	Off-line SPE-UPLC–MS/MS		Off-line μSPE-UPLC–MS/MS	
	10-fold dilution	100-fold dilution	10-fold dilution	100-fold dilution
CAR	6.5 ± 0.3 (3.7)	6.2 ± 0.0 (0.1)	8.0 ± 0.0 (0.6)	10.7 ± 0.5 (4.6)
ANS	9.5 ± 0.0 (0.2)	7.9 ± 0.2 (2.6)	9.3 ± 0.2 (2.1)	10.7 ± 0.4 (4.0)

pre-treatment with those obtained from standard solutions at different concentrations.

Both a negative and a positive effect was observed in the two methodologies developed (off-line SPE and μSPE-UPLC–MS/MS), which meant a decrease and an increase in the detector response, respectively. When the HLB sorbent (both μSPE and SPE) was used, the matrix effects were low and similar for the two dipeptides, from −9 to 12% (Table 2). The matrix effect was negative (a decrease in the detector response) for the analysis of CAR and positive (an increase in the detector response) for the analysis of ANS. On the other hand, when both the ion-exchange sorbents, WCX and MAX, were used, the matrix effect was higher, between 15 and 44%.

In conclusion, although the HLB (both μSPE and SPE) and WCX sorbents gave the same extraction recoveries (80–90%), the ion-exchange WCX and MAX sorbents were discarded as the sample pre-treatment strategies for the analysis of chicken broth, due to the high matrix effect. Therefore, the HLB sorbent with the two device formats, μSPE and SPE, was used to determine CAR and ANS in the chicken broth, and then the results obtained were compared.

3.3. Quality parameters of the developed method

Once the improved analytical method was developed, its instrumental quality parameters were determined by spiking the diluted (100-fold) chicken broth extract with CAR and ANS at different known concentrations. The instrumental quality parameters were linearity range, extraction recovery, calibration curve, repeatability, accuracy, robustness, the LOD and the LOQ. These parameters were studied by using both off-line SPE and off-line μSPE with HLB sorbent, as the sample pre-treatment strategy, in order to compare the results. The results obtained are shown in Table 2. The 100-fold dilution was used as a blank to study the quality parameters of the method. As in this dilution both CAR and ANS were detected, the real concentrations of these analytes were subtracted from the blank concentration.

The linearity range of the analytical method was evaluated by using the diluted chicken broth extract spiked with the dipeptides, as previously reported. By using off-line μSPE as the sample pre-treatment, the linearity was from 7 to 3300 μg/l for both CAR and ANS. On the other hand, when off-line SPE was used, the linearity range was slightly lower, from 2 to 1000 μg/l for both CAR and ANS. The calibration curves (obtained based on the integrated peak area) were calculated by using six points at different concentrations levels, and each concentration was injected three times. Table 2 also shows the determination coefficient (R_2) of the calibration curves, and these values were higher than 0.994.

The repeatability of the improved method was determined by the relative standard deviation (% RSD), in terms of concentration, and this was measured at five concentration levels, from 10 to 3300 μg/l for off-line μSPE and from 4 to 1000 μg/l for off-line SPE. The RSDs were similar for the two developed methodologies and ranged from 1.7 to 14.6%. The accuracy was calculated from the ratio between the concentration of the dipeptides found compared with the spiked concentration. This quotient was then multiplied by 100. This quality parameter was studied at three concentration levels, these being 40, 200 and 1000 μg/l, and the accuracy was good, from 95 to 105%, in the two SPE methodologies.

The robustness of the developed method was evaluated by varying the operational parameters of the method within a reasonable range. These operational parameters of the method were concentration of the mobile phase, pH of the mobile phase, flow-rate, column temperature, injection volume, and also, the device format of the sample pretreatment, cartridges or microplates. Although, these parameters were varied within a reasonable range, the concentration of the studied bioactive dipeptides was the same. Therefore, the developed method can be defined as robust and reliable.

The LODs and LOQs, calculated using the signal-to-noise ratio criterion of 3 and 10, respectively, were at low μg/l concentration levels. The LOQs of ANS and CAR were 2 μg/l and 6 μg/l depending on whether the sample pre-treatment methodology was off-line SPE or μSPE, respectively. Similarly, the LODs of ANS and CAR were 0.6 μg/l and 1.8 μg/l for the off-line SPE or μSPE, respectively (Table 2). When the SPE sample pre-treatment was used, the LODs and LOQs were slightly lower than when μSPE was used. This fact was explained by the higher sample amount loaded into the cartridge (1 ml) when SPE was used than with μSPE (350 μl). The obtained results (LODs and LOQs) were lower than those reported in the literature for the determination of CAR and ANS in meat and feed samples by UV [14,18,20], amperometric [21] and MS [19]. Therefore, the developed method was improved respect to those reported in the literature for the analysis of CAR and ANS in sensitivity.

As it has been previously reported, the extraction recovery of CAR and ANS was similar and excellent, ranging from 83 to 98%. This extraction recovery corresponded to the SPE and μSPE extractions. On the other hand, the extraction recovery was also determined in all the process (centrifugation and μSPE or SPE). Therefore, the responses of the dipeptides spiked in the chicken broth before and after extraction were compared. This %*R* was slightly lower than the %*R* from SPE and μSPE, and it could be due to the dipeptides being lost in the glass wool when the supernatant was filtered. This %*R* was between 62 and 75% for the two dipeptides.

In conclusion, the quality parameters of the method by using both traditional cartridges SPE and μSPE plates as the sample pre-treatment strategy were similar.

3.4. Application of the improved developed method

The improved developed methods (off-line SPE and off-line μSPE) were applied for the determination of CAR and ANS in commercial chicken broth. Fig. 2 shows the total ion chromatograms (TIC) (A) and the extracted ion chromatograms obtained to determine CAR (B) and ANS (C) in this sample. These chromatograms were obtained by using the 100-fold dilution and off-line SPE with traditional cartridges as the sample pre-treatment. Although the TIC shows a little overlapping between the peaks of both dipeptides, the matrix effect was also studied and this it was low, also from −9 to 12% for the two dipeptides. Therefore, the developed method is reliable to quantification purposes.

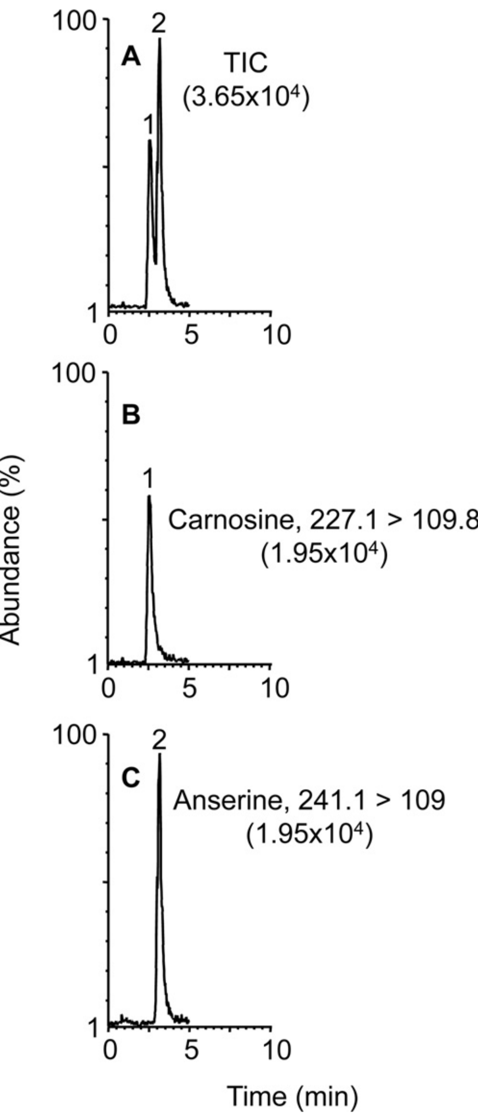

Fig. 2. Total ion chromatogram (TIC) (A) and extracted ion chromatograms obtained for the determination of CAR (B) and ANS (C) in chicken broth.

Table 3 shows the concentration (mg/l) ± standard deviation (s) of CAR and ANS in chicken broth when both 10-fold and 100-fold dilutions, and SPE and μSPE as sample pre-treatment, as the device format, were used. By comparing these results (concentrations) obtained from the two dilutions, for the analysis of CAR and ANS, no significant differences were observed. On the other hand, when the sample pre-treatments SPE and μSPE used to determine CAR and ANS in chicken broth were compared, it was revealed that these sample pre-treatment strategies had a significant effect. As can be seen in Table 3, the concentrations of CAR and ANS when analyzing the chicken broth by μSPE were higher than when these dipeptides were analysed by SPE. CAR was determined at around 6 mg/l and 9 mg/l depending respectively on whether the SPE or μSPE sample pre-treatment was used. For the analysis of ANS, this dipeptide was determined around 8.5 mg/l and 10 mg/l according to the SPE and μSPE sample pre-treatment, respectively, used.

4. Conclusions

This paper describes a rapid, sensitive and efficient method for the analysis of the bioactive dipeptides CAR and ANS compared with those reported in the literature. These improvements have been achieved by the use of a stationary phase packed with 1.7 μm instead of 5 μm particles, and the use of tandem MS as the detection system instead of UV. The developed methodology allowed CAR and ANS to be determined in a short analysis time with LODs between 1.8 and 0.6 μg/l depending on whether the device format was μSPE or SPE, respectively.

For the analysis of chicken broth, both SPE and μSPE with HLB sorbent were satisfactory used to extract the studied dipeptides from the salt with extraction recoveries between 80% and 90%, and with low matrix effect, from −9% to 12%. The quality parameters of the developed methods were similar when using both traditional cartridges SPE and μSPE plates, as the sample pre-treatment strategy. CAR and ANS were determined at low mg/l concentration levels. No significant effect was shown on the analysis of CAR and ANS in chicken broth when comparing the 10-fold and 100-fold dilution. In contrast, a significant effect was shown when the SPE and μSPE sample pre-treatments were used to analyse CAR and ANS in chicken broth. Therefore, the developed methods allow polar analytes to be determined satisfactorily and rapidly by MS as the detection system in samples that contain salt.

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