USING OF ELSD FOR DETERMINATION OF ORGANIC ENGINEERED NANOPARTICLES

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Abstract

In presented study determination of various organic engineered nanoparticles ENPs, with cross-linked gelatine and/or polysorbate based nanoparticles in water and beverages was examined. For this purpose Ultra Performance Liquid Chromatography (UPLC) Size Exclusion Chromatography (SEC) coupled with Evaporative Light Scattering Detector (ELSD) was used. Analytical method for characterisation and determination of nanoparticles in food matrices was developed; sample preparation procedure and parameters of determination of ENPs were optimised to achieve the best performance characteristics. For cross-linked gelatine various storage and hydrolysis conditions were tested to verify the stability of prepared cross-linked gelatine. The limit of quantification (LOQ) of organic ENPs was: (i) for cross-linked gelatine 1 mg/mL in beverages and 0.5 mg/mL in water and (ii) for polysorbate based ENPs 0.5 mg/mL in beverages and 0.1 mg/mL in water.

Keywords: organic engineered nanoparticles, cross-linked gelatine, polysorbates, UPLC-SEC-ELSD, beverages

1 INTRODUCTION

The interest in application of nanoparticles in various industry branches has increased explosively over the past two decades [1]. In food sector, application of nanotechnologies - derived food ingredients, additives, supplements, and contact materials is expected to grow continuously [2, 3, 4]. In many cases, they are built from safe materials such as polysaccharides or lipids which are encapsulating respective biologically active compound to be used as food additive. Micelles represent another common nano carrier system [5]. To date, little is known about the occurrence, fate and toxicity of nanoparticles. Detection and characterisation of nanoparticles are essential in understanding of the benefits as well as potential risks of the application of such materials in food. Analytical methods have been developed for inorganic nanoparticles in simple food matrices. However, methods for separation and detection of organic engineered nanoparticles (ENPs) in foodstuffs and nutraceuticals are still under development and, in general terms are lacking. There are published few recent reviews on the detection of organic nanoparticles in food [1, 6, 7, 8]. To get a new knowledge on food ENPs nanoparticle properties and strategies applicable for their control in food NanoLyse (www.nanolyse.eu) EU 7th FP project (Nanoparticles in Food: Analytical methods for detection and characterisation) was established [1]. As regards analysis, several tasks including ENPs isolation and discrimination from the sample matrix have to be solved. Chromatography and/or mobility analyzers may be used for size separation and collection of fractions for further instrumental analysis. In the next step, identification/detection and possibly quantification of either encapsulate material or directly active ingredient should be performed; typically mass spectrometry is employed for this purpose. European Commission (2011) [9] published its recommendation on the definition of nanomaterials. Thus, to detect the presence of nanomaterials in food according to this definition requires analytical methods, able to determine the number size distribution of particles at least in the 1-100 nm size range [8]. Recognising the developments in the field and the possible applications of nanotechnology to the food sector the European Food Safety Authority (EFSA) published in 2011 the first practical guidance for assessing nano applications in food and feed [10].
2 EXPERIMENTAL

2.1 Tested material
Organic ENPs based on Polysorbate 20 and Polysorbate 80 were obtained from the EC-JRC-IRMM (Geel, Belgium). Cross-linked gelatine nanoparticles were prepared within the EU 7th FP NanoLyse project network (www.nanolyse.eu). Reference biopolymers - porcine and bovine gelatine and Polysorbate 20 and 80 were obtained from Sigma-Aldrich, Germany. Apple and orange juice and Aquarius drink for model experiments were purchased from retail market.

2.2 UPLC-SEC-ELSD analysis
For detection of organic ENPs the UPLC system (Acquity Waters, USA) coupled with the Evaporative Light Scattering Detector (ELSD, Waters, USA) was used. The separation was carried out with the ACQUITY UPLC® BEH200 SEC analytical column (150 x 4.6 mm; 1.7 µm). The flow rate of mobile phase (5mM or 50nM ammonium formate) was 0.3 mL/min, column temperature 35°C, injection volume 2 µL and injector temperature 23°C. The optimized ELSD conditions used for detections were as follows: temperature of drift tube 50°C, nebulizer temperature 12°C, gain 500, and nitrogen pressure 40 psi.

3 RESULTS AND DISCUSSION
As mentioned in Introduction, analysis of organic ENP is not an easy task. Chromatographic separation from other sample components might be a feasible approach. In the presented study a potential of UPLC-SEC employing size exclusion analytical column BEH200 for separation of ENPs was demonstrated. To monitor elution of target compounds, Evaporative Light Scattering detector (ELS) was employed. The parameters of ELS detection were optimised, final set-up used for all experiments is described in Experimental part. For UPLC separation at SEC column, an efficiency of mobile phase containing volatile ammonium formate in different concentrations was tested. The differences between 5 mM and 50 mM ammonium formate concentration in sample separation were not observed, therefore the lower concentration was used in all experiments.

3.1 Cross-linked gelatine
The possibility to determine both cross-linked gelatine produced within the NanoLyse project in water and beverages (apple and arrange juice, and Aquarius drink) and commercially purchased porcine and bovine gelatine was examined. The peak of cross-linked gelatine was eluted under described analytical conditions at retention time (RT) approx. 2.85 min (Fig. 1). The response for cross-linked gelatine in water as well as in tested beverages was linear in the concentration range 0.5 – 40 mg/mL. When porcine and bovine gelatin were analysed, unfortunately, no peaks at elution zone of high molecular compound were noticed under applied elution and detection conditions. The limit of quantification (LOQ) of cross-linked gelatine in water was 0.5 mg/mL and in beverages 1 mg/mL. As shown in Fig. 1, non high molecular matrix interferences were present in pure orange juice (only low molecular size compounds were eluted in the retention times range 5.5 – 7.0 min), thus cross-linked gelatine could be selectively detected (Fig. 1).

All tested samples were filtered before UPLC-SEC analysis through 0.45 µm pores’ size membrane filters, only cross-linked gelatine with particle size higher than 500 nm was filtered through 5 µm pores’ size filters due to possible losses during filtration through 0.45 µm filters.

The stability of cross-linked gelatine was also tested to check the influence of various conditions on its levels in beverages. In the first step, the influence of sonication and boiling on the stability of cross-linked gelatine was investigated. Only relatively small decrease of gelatine response was found after sonication and boiling; the stability was higher in water solution in comparison with juices. Remaining content of cross-linked
gelatine in apple juice was 93% after sonication and 79% after boiling and in water it was 93% for both tested procedures.

![UPLC–SEC-ELSD analysis of A) orange juice and B) cross-linked gelatine at level 5 mg/mL in orange juice](image)

**Fig. 1:** UPLC–SEC-ELSD analysis of A) orange juice and B) cross-linked gelatine at level 5 mg/mL in orange juice

The stability of cross-linked gelatine in solutions (concentrations 1, 5, 10 mg/mL) during storage was better in water compared to orange juice, more concentrated solutions were more stable. After 17 days of storage at 4°C the decrease of cross-linked gelatine content for solutions at original levels 1, 5 and 10 mg/mL was 87%, 51%, and 37% in orange juice and 38%, 34%, and 32% in water, respectively.

The effect of enzymatic digestion by proteolytic enzyme trypsin when employing various incubation times and enzyme concentration was examined to assess the stability of cross-linked gelatine. For hydrolysis different volume (200, 400, 600 and 1000 µL) of trypsin (1mg/mL in 0.05 M NH₄HCO₃ pH 8) were added, the incubation temperature was 37°C and the time of hydrolysis was 2, 3, 4 and 16 hours. Cross-linked gelatine was shown to be relatively stable against hydrolysis; the most efficient hydrolytic conditions were 600 µL of trypsin and 4 hours of hydrolysis. The effect of enzymatic hydrolysis on cross-linked gelatine is demonstrated at **Fig. 2.** The influence of acidic hydrolysis (4 h, 100°C, 6 M HCl) on stability of cross-linked gelatine was tested too; the polymer was more stable under these conditions compared to enzymatic hydrolysis.

It should be noted, that by tested analytical column was not able to separate with sufficient resolution cross-linked gelatine produced within the NanoLyse project according to molecule size. There are only small differences in RT of peaks obtained for cross-linked gelatine at different size (183 nm – RT 2.888 min; 550 nm – RT 2.818 min). The chromatogram at **Fig. 3** illustrates separation of different size cross-linked gelatine in Aquarius drink. However, separation from food components was sufficient to determine cross-linked gelatine content in a range of beverages. For better separation of different size nanoparticles, SEC column with more appropriate column exclusion limit for the range of tested nanoparticles and its size should be used.
Fig. 2: UPLC–SEC-ELSD analysis of cross cross-linked gelatine 17 mg/mL in water A) before hydrolysis, B) after hydrolysis, 4 hours – 200 µl trypsin, and C) 4 hours – 600 µl trypsin

Fig. 3: UPLC–SEC-ELSD analysis of cross-linked gelatine in Aquarius drink A) concentration 6 mg/mL, size 183 nm and B) concentration 16 mg/mL, size 550 nm (green frame shows elution zone of different size cross-linked gelatine, a red frame shows elution of juice components)
3.2 Polysorbate based ENPs

UPLC-SEC-ELSD technique was also tested for determination of Polysorbate 20 and 80 and polysorbate based nanoparticles, creating micelles, which can be used as carrier for lipophilic or water-insoluble substances. When pure polysorbates were analysed, two peaks at retention times 6.0 and 6.3 min (probably polysorbate dimmers and monomers, respectively) were detected.

Polysorbate based nanoparticles in solution form micelles with size 10 – 30 nm. These micelles are eluted at RT 3.0 min (Fig. 4), however parts of “free polysorbates” are still eluted at RT 6.0 and 6.3 min.

Before the analysis, an effect of filter used for sample filtration was tested; membrane filters with porosity 5 µm, 0.45 µm a 0.22 µm were compared. Pores 5 µm are too high for UPLC analysis and do not allow removing of matrix particles. When filters 0.45 µm and 0.22 µm were applied, there were no differences found for pure polysorbates, however for polysorbate based ENPs the losses of responses for micelles were significant when 0.22 µm filters were used. Based on these findings, all tested samples with pure polysorbates and polysorbate based nanoparticle were filtered through 0.45 µm filters before UPLC analysis.

Calibration curves for Polysorbate 20 and 80 and also polysorbate based ENPs were linear in the range 0.1 mg/ml - 10 mg/ml of water. When orange juice with addition of polysorbate based ENPs was analysed, only peak of micelles can be separated from low molecular compounds originating from juice, the peaks at RT 6.0 and 6.3 min were co-eluted with juice low molecular fraction. For more reliable measurement of added ENPs concentration, matrix-matched calibration is recommended; limit of quantification (LOQ) in orange juice was 0.5 mg/mL. This method can be used for analysis of organic engineered nanoparticles (ENPs) based on polysorbates in beverages, by this method we cannot separate Polysorbate 20 and 80.

![UPLC–SEC-ELSD analysis of A) Polysorbate 20 and B) organic ENPs based on Polysorbate 20; concentration of both 1 mg/mL in water](image)

Fig. 4: UPLC–SEC-ELSD analysis of A) Polysorbate 20 and B) organic ENPs based on Polysorbate 20; concentration of both 1 mg/mL in water

4 CONCLUSION

Application potential of UPLC–SEC-ELSD method for monitoring of ENPs in water and beverages (fruit juices and Aquarius drink) was tested. It is possible to distinguish nano materials from low molecular...
compounds originating from food matrices by this method. Whereas the ELSD detection is not selective, there was not possible to distinguish between the different “high size” molecules and identified them. This approach is suitable as supplementary method to any selective and sensitive analytical procedure (e.g. mass spectrometric detection) and is useful for rapid identification and determination of “nano” size components of food matrices. For quantification matrix matched calibrations are more specific compared to aqueous solutions.

The limit of quantification (LOQ) of organic ENPs is: (i) for cross-linked gelatine 1 mg/mL in beverages and 0.5 mg/mL in water and (ii) for polysorbate based ENPs 0.5 mg/mL in beverages and 0.1 mg/mL in water.

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LITERATURE


