

# Analytical strategies for controlling polysorbate-based nanomicelles in fruit juice

Veronika Krtkova · Vera Schulzova · Ondrej Lacina ·  
Vojtech Hrbek · Monika Tomaniova · Jana Hajslova

Received: 21 February 2014 / Revised: 3 April 2014 / Accepted: 4 April 2014 / Published online: 9 May 2014  
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**Abstract** This study focused on the detection and quantification of organic micelle-type nanoparticles (NPs) with polysorbate components (polysorbate 20 and polysorbate 80) in their micelle shells that could be used to load biologically active compounds into fruit juice. Several advanced analytical techniques were applied in the stepwise method development strategy used. In the first phase, a system consisting of ultrahigh-performance liquid chromatography employing a size exclusion column coupled with an evaporative light scattering detector (UHPLC-SEC-ELSD) was used for the fractionation of micelle assemblies from other, lower molecular weight sample components. The limit of detection (LoD) of these polysorbate micelles in spiked apple juice was  $500 \mu\text{g mL}^{-1}$ . After this screening step, mass spectrometric (MS) detection was utilized to confirm the presence of polysorbates in the detected micelles. Two alternative MS techniques were tested: (i) ambient high-resolution mass spectrometry employing a direct analysis in real time ion source coupled with an Orbitrap MS analyzer (DART-Orbitrap MS) enabled fast and simple detection of the polysorbates present in the samples, with a lowest calibration level (LCL) of  $1000 \mu\text{g mL}^{-1}$ ; (ii) ultrahigh-performance reversed-phase liquid chromatography coupled with high-resolution time-of-flight mass spectrometry (UHPLC-HRTOF-MS) provided

highly selective and sensitive detection and quantification of polysorbates with an LCL of  $0.5 \mu\text{g mL}^{-1}$ .

**Keywords** Micellar nanoparticles · Polysorbate 20 and 80 · Apple juice · UHPLC-SEC-ELSD · DART-Orbitrap MS · UHPLC-HRTOF-MS

## Introduction

Interest in the application of nanoparticles (NPs) in various branches of industry has greatly increased over the past two decades [1]. In the food sector, applications of nanotechnologies, such as derived food ingredients, additives, supplements, and contact materials, are expected to grow continuously [2–4]. In addition to NPs that are generated from inorganic materials, NPs that only contain organic molecules have also become available. These are built from “safe” materials such as polysaccharides, proteins, and lipids, which in turn encapsulate biologically active compounds that are intended for use in a targeted context. Nanoencapsulation in the form of micelles is one of the nanodelivery systems available for nutrients and preservatives [5]. Although the prospective beneficial effects of nanotechnologies and NPs have been thoroughly described in general, studies assessing their potential toxicological effects and impacts are still scarce [5]. To date, many in vitro toxicological studies on inorganic NPs have been reported, but little is known about the occurrence, fate, and toxicity of organic NPs [6–10]. It should be noted that it is essential to be able to detect and characterize nanoparticles if we are to understand the benefits and potential health risks associated with the applications of such materials in food.

Recently, the European Commission published recommendations for defining nanomaterials: to detect their presence in food, analytical methods are required that enable the determination of the particle size distribution in the range of 1–

Published in the topical collection *Characterisation of Nanomaterials in Biological Samples* with guest editors Heidi Goenaga-Infante and Erik H. Larsen.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00216-014-7823-7) contains supplementary material, which is available to authorized users.

V. Krtkova · V. Schulzova (✉) · O. Lacina · V. Hrbek ·  
M. Tomaniova · J. Hajslova (✉)  
ICT Prague, Technicka 3, 166 28 Prague, Czech Republic  
e-mail: vera.schulzova@vscht.cz  
e-mail: jana.hajslova@vscht.cz

100 nm at least [11–13]. The annual report of the European Food Safety Authority (EFSA) defined nanomaterials as a natural, incidental, or manufactured material that contains particles in an unbound state or as an aggregate or agglomerate, and 50 % or more of the particles in the number size distribution have one or more external dimensions in the size range 1–100 nm. In specific cases, and when warranted by concerns about the environment, health, safety, or competitiveness, this number size distribution threshold of 50 % may be replaced by a smaller threshold, possibly as low as 1 % [13]. The EU (in regulation no. 1169/2011) recently decided that food labelling for consumers should include information on any nanomaterials present in the food. In 2011, the EFSA published the first practical guidance on the assessment of engineered nanomaterials in food and feed [14]. The European Committee for Standardisation and Research (CEN-STAR) summarized progress in standardization in the field of engineered nanoparticles. The main items for standardization include: definition of the terminology, instrumentation methodology, reference methods and materials, measurement methods for applications (physical, chemical, material, electronics), and measurement methods for use in the fields of health, the environment, and safety [15]. Information—which is rather scarce—on the standardization of nanotechnology in Europe includes the Code of Conduct for Responsible Nanosciences and Nanotechnologies Research as well as the European Commission mandate to CEN/TC 352 [16].

Currently, several companies (e.g., Zymes, Aquanova, Nutralease, Solgar) produce nanosized micelles that are available for various applications in food-production materials, food processing, and food products. These commercial micelles are typically around 30 nm in size, so they can be regarded as “micellar nanoparticles” [1, 17–19].

To date, most analytical methods for use in this field have been developed for inorganic nanoparticles in simple food matrices. Recently, a few reviews on conceivable approaches for the detection of organic nanoparticles (NPs) in food have been published [1, 11, 20, 21]. Microscopy and microscopy-related techniques (electron microscopy and scanning probe microscopy) are the most popular methods employed for the characterization and visualization of nanoparticles (NPs), while chromatography and other separation techniques can be used to separate NPs in complex samples. In addition to size-exclusion chromatography (SEC), hydrodynamic chromatography (HDC) is a widely used technique for separating particles in the nanometer range on the basis of size. Field-flow fractionation (FFF) is another highly promising technique for the separation of high molar mass analytes, from macromolecules to NPs (nm range) and  $\mu\text{m}$ -sized particles [1]. In FFF, unwanted interactions of nanoparticles with the stationary phase are avoided, meaning that this separation method is relatively gentle, thus allowing the analysis of more delicate samples [22]. SEC, HDC, and FFF can be combined

with detection modes such as UV/VIS, dynamic light scattering (DLS), evaporative light scattering (ELS), multi-angle laser light scattering (MALLS), and mass spectrometry (MS). These techniques can be successfully used for the detection and quantification of NPs composed of protein, lipid, and/or polysaccharide blocks [1, 20, 23]. However, it should be noted that methods for separating and detecting organic NPs in foodstuffs and nutraceuticals are still under development and are, in general, rather limited. To gain new knowledge on the properties of NPs and strategies for controlling their levels in food, reliable analytical methods of detecting and quantifying NPs must be developed. In particular, isolating NPs and distinguishing them from the sample matrix are difficult tasks that have to be solved. Selective detection is complicated by the similarities of the chemical structures of the building blocks of organic NPs to the food matrices in which they are incorporated, as well as the tendency of NPs to disassemble during the course of sample preparation.

The study reported in the present paper focused on methods for monitoring polysorbate 20 and/or polysorbate 80 micelles, which are used as nanocarrier systems for delivering nutraceuticals, vitamins, etc. into aqueous matrices. Polysorbate 20 and 80 are amphiphilic, nonionic surfactants composed of fatty acid esters of polyoxyethylene sorbitan. Polysorbate 20 (polyoxyethylene sorbitan monolaurate) and polysorbate 80 (polyoxyethylene sorbitan monooleate) are the polymers of this category most commonly used in formulations of protein biopharmaceuticals, and are often used in foodstuffs as emulsifiers and stabilizers. Commercially available polysorbate 20 and polysorbate 80 are chemically diverse mixtures containing mainly fatty acid esters of polyoxyethylene sorbitan [24, 25]. Polysorbates are commonly used in formulations above their critical micelle concentration (CMC) values. However, at concentrations below their CMCs, polysorbate micelles are unstable and decompose [26]. In the EU, polysorbates 20, 40, 60, 65, and 80 were approved for use in food by the Directive on Food Additives (1995), and standards for their use have been established. Commission Regulation (EC) no. 1129/2011 established maximum levels of polysorbates in foodstuffs [27, 28]. An acceptable daily intake (ADI) of  $10 \text{ mg kg}^{-1} \text{ bw/day}$  of polysorbates was allocated by the Scientific Committee on Food [29]. Techniques for determining free polysorbate contents are available, such as nuclear magnetic resonance (NMR), UV/VIS, high-performance liquid chromatography coupled with condensation nucleation light-scattering detection (HPLC-CNLS), and high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) [26, 30–32]. However, techniques for determining polysorbates in micellar form have not yet been published.

Several analytical approaches have been combined in this study. While DART-Orbitrap MS was used to screen for the presence of polysorbates, UHPLC-HRTOF-MS was

employed for their detection and quantification. The UHPLC-SEC-ELSD technique was used to verify that high molecular weight components such as polysorbate (PS) micelles were present in juice samples.

## Experimental

### Experimental samples

The following commercially available nanomicelles with a polysorbate 20/80 shell and encapsulated nutrients and/or preservatives were tested: (i) vitamin E solubilize 20 %, (ii) glucose solubilize 5 %, (iii) medium-chain triglyceride solubilize 10 %. This nanomaterial was provided by the NanoLyse EU project (<http://www.nanolyse.eu>) partner EC-JRC-IRMM (Geel, Belgium).

### Reagents and chemicals

Standards of polysorbates 20 and 80, DL- $\alpha$ -tocopherol acetate (purity  $\geq 96\%$ ), and caffeine (anhydrous, purity  $\geq 99.0\%$ ) used as an internal standard were obtained from Sigma-Aldrich (Germany). Methanol (HPLC grade) and ammonium formate (for HPLC, purity  $\geq 99.0\%$ ) were also obtained from Sigma-Aldrich. Ultrapure water was produced by a Milli-Q system (Millipore Corporation, Bedford, MA, USA). Apple juice (100 % juice) was purchased from the retail market. Sample solutions were passed through 0.45- $\mu\text{m}$  PVDF microfilters (National Scientific, Rockwood, TN, USA).

### Characterization of polysorbate micelles

A Zetasizer Nano-ZS instrument (Malvern Instruments, Malvern, UK) was employed to verify that the tested polysorbate (PS) micelles were nanosized. For the size measurements, 30 mg of the tested PS micelles were mixed with 10 mL of water. An aliquot of 1 mL of each sample was filtered through a 0.45- $\mu\text{m}$  PVDF filter and transferred to the measuring cuvette for dynamic light scattering measurements.

### Sample preparation for the validation study

The sample preparation procedure employed depended on the instruments employed for free polysorbate and polysorbate (PS) micelle detection/quantification. In all cases, matrix-matched standards in filtered apple juice were prepared for calibration, and six replicate measurements were made. In regards to defining an equivalent to the limit of quantification (LoQ), performance characteristics are commonly employed in MS/MS. However, the “classic” definition, based on the signal-to-noise ratio (typically  $S/N > 6$ ), is not always applicable to high-resolution MS detection because chemical noise

can be absent from a chromatogram. Because of this, we decided that the lowest calibrated level (LCL) approach was the most suitable option.

*Ultra-high-performance size-exclusion chromatography coupled with an evaporative light-scattering detector (UHPLC-SEC-ELSD)* Ten milliliters of apple juice were spiked with the PS micelles (concentration level  $10\text{ mg mL}^{-1}$ ) and polysorbate 20/80 (concentration level  $10\text{ mg mL}^{-1}$ ). To avoid clogging the UHPLC analytical column containing 1.7- $\mu\text{m}$  sorbent particles, an aliquot of 1 mL of each sample was filtered through the 0.45- $\mu\text{m}$  PVDF microfilter and then transferred to the LC vial. It was verified that no losses of nanomicelles occurred when using this filter.

*Direct analysis in real time ion source coupled with an Orbitrap mass analyzer (DART-Orbitrap MS)* Ten milliliters of apple juice were spiked with PS micelles (two concentration levels:  $10\text{ mg mL}^{-1}$  and  $5\text{ mg mL}^{-1}$ ) and polysorbate 20/80 (two concentration levels:  $10\text{ mg mL}^{-1}$  and  $5\text{ mg mL}^{-1}$ ), and caffeine was used as an internal standard ( $10\text{ }\mu\text{g mL}^{-1}$ ). An aliquot of 1 mL of each sample was filtered through the 0.45- $\mu\text{m}$  PVDF microfilter and transferred to the LC vial. This procedure was performed for six replicates.

*Ultra-high-performance liquid chromatography coupled with time-of-flight mass spectrometry (UHPLC-HRTOF-MS)* Ten milliliters of apple juice were spiked with PS micelles (three concentration levels:  $100\text{ }\mu\text{g mL}^{-1}$ ,  $50\text{ }\mu\text{g mL}^{-1}$ , and  $10\text{ }\mu\text{g mL}^{-1}$ ) and polysorbate 20/80 (three concentration levels:  $100\text{ }\mu\text{g mL}^{-1}$ ,  $50\text{ }\mu\text{g mL}^{-1}$ , and  $10\text{ }\mu\text{g mL}^{-1}$ ). An aliquot of 1 mL of each sample was diluted in 9 mL methanol, filtered through the 0.45- $\mu\text{m}$  PVDF microfilter, and transferred to the autosampler vial prior to LC analysis. This procedure was performed for six replicates. When the PS micelle concentration in the juice sample was expected to be too high, dilution with a water:methanol (1:9, v/v) mixture was performed to get the concentration within the calibration range.

### Instrumentation and experimental conditions

*UHPLC-SEC-ELSD system* This system consisted of UPLC chromatograph (ACQUITY, Waters, Milford, MA, USA) coupled with an evaporative light-scattering detector (ELSD, Waters). The separation was carried out with an ACQUITY UPLC® BEH200 SEC column ( $150 \times 4.6\text{ mm}$ ;  $1.7\text{ }\mu\text{m}$ ). The flow rate of the mobile phase (5 mM ammonium formate) was  $0.3\text{ mL min}^{-1}$ , the column temperature was  $35\text{ }^\circ\text{C}$ , the injection volume was  $2\text{ }\mu\text{L}$ , and the injector temperature was  $23\text{ }^\circ\text{C}$ . The optimized ELSD conditions used for detection were as follows: temperature of drift tube  $50\text{ }^\circ\text{C}$ , nebulizer temperature  $12\text{ }^\circ\text{C}$ , gain 500, and nitrogen pressure 40 psi.

**DART-Orbitrap MS system** This system consisted of a DART ion source (IonSense, Saugus, MA, USA) coupled with an Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) and a 12 Dip-it scanner autosampler (IonSense). Helium was used as the ionization gas. The operating conditions of the DART ion source were: positive ion mode; helium temperature 300 °C; input pressure of ionization gas 4.5 bar; autosampler sampling speed 0.6 mm s<sup>-1</sup>; desorption time 5 s. The operating conditions of the Orbitrap MS were: capillary voltage: +60 V; tube lens voltage +140 V; capillary temperature 250 °C; monitored mass range:  $m/z$  100–1100; in-source CID 25 eV; resolving power 100,000 FWHM at  $m/z$  200. Sample insertion was carried out automatically using Dip-it™ samplers. The glass sampling rod was immersed for 1 s in the sample well of a deep-well microplate (Life Systems Design, Merenschwand, Switzerland) and transferred to the optimized position in front of the DART source exit. The sample was then desorbed from the surface of the glass rod for 5 s by hot helium, which instantaneously ionized the sample, while the MS spectral data were recorded in real time.

**UHPLC-HRTOF-MS system** This system consisted of a UHPLC chromatograph (ACQUITY UPLC, Waters) coupled with a high-resolution time-of-flight mass spectrometer (Synapt G2 HDMS, Waters). The separation was carried out on a reversed-phase ACQUITY UPLC® HSS T3 (100 × 2.1 mm and 1.8 μm particle) analytical column maintained at 40 °C. The mobile phase consisted of 5 mM ammonium formate in Milli-Q water (A) and methanol (B). The initial composition (90 % A, flow rate 0.3 mL min<sup>-1</sup>) was maintained for 0.5 min. The mobile phase composition was changed linearly to 0.1 % (A) up to 8 min, and the flow rate was simultaneously changed from 0.3 to 0.6 mL min<sup>-1</sup>. This mobile-phase composition was maintained up to 13 min, and

column reconditioning with the initial composition was then performed for the next 3 min. The injection volume was 5 μL, and the partial loop pressure assist mode was employed. These conditions were tested beforehand for the UHPLC-ELSD system. The operating parameters of the HRTOF MS with an electrospray interface operating in the positive ion mode were as follows: capillary voltage 3,000 V; cone voltage 40 V; desolvation temperature 350 °C; source temperature 120 °C; cone gas 50 L h<sup>-1</sup>; desolvation gas 800 L h<sup>-1</sup>. The leucine-enkephalin lock mass calibrant (flow 20 μL min<sup>-1</sup>) was recorded every 40 scans. Full-scan spectra from 100 to 2,000 Da were acquired in the resolution mode with a resolving power of more than 20,000 FWHM at  $m/z$  556.2766 (leucine-enkephalin).

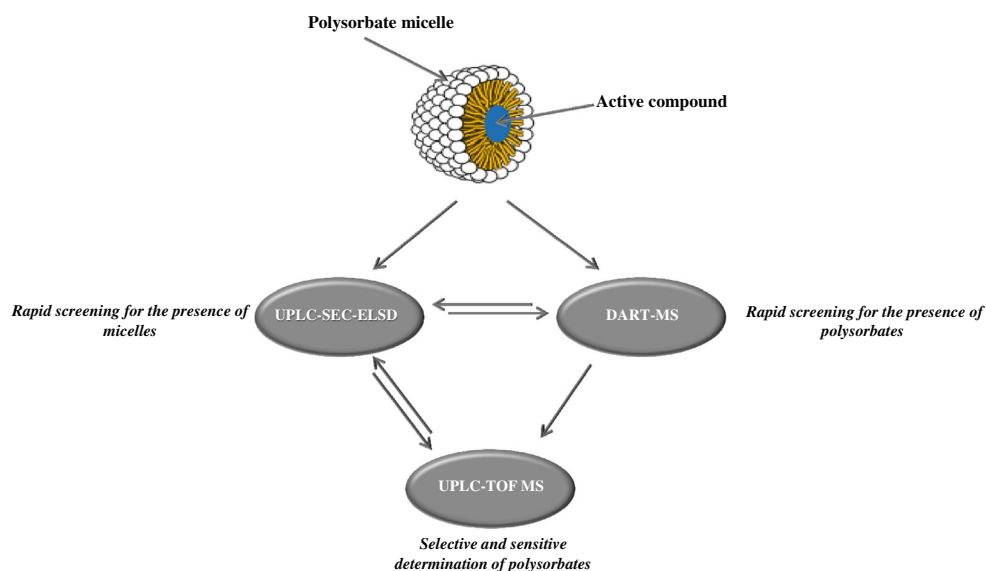
## Results and discussion

As emphasised in the “Introduction,” the analysis of polysorbate micelles in food matrices is a challenging task because, in many cases, the components in the PS micelle sphere are similar to those naturally occurring in the matrix in which they are incorporated. Moreover, the use of organic solvents during sample preparation and sample separation must be minimized to preserve their assembled state.

In the current study, the target analytes were micelles based on polysorbate 20/80 (i.e., polymers that are not a common food component), so the implementation of a selective detection strategy was enabled. An outline of the various analytical approaches employed in this study is provided in Fig. 1. The development of the individual instrumental methods used here is described in more detail in the paragraphs below.

In the first step, the polysorbate micelle size was characterized using a dynamic light-scattering technique (Zetasizer Nano-ZS). The z-average diameters of the polysorbate

**Fig. 1** Strategy for detecting and determining PS micelles in fruit juice





micelles are shown in Fig. S1 of the “Electronic supplementary material,” ESM. The following z-average diameters were obtained for the tested PS micelles with encapsulated nutrients and preservatives: (i) vitamin E solubilizate 20 %,  $28.8 \pm 0.2$  nm; (ii) glucose solubilizate 5 %,  $10.9 \pm 0.9$  nm; (iii) medium-chain triglyceride solubilizate 10 %,  $30.2 \pm 0.5$  nm.

Fruit juice was chosen as a demonstration matrix within the NanoLyse project since it can only be enriched with lipophilic components (e.g., fat-soluble vitamins), which may be necessary in certain situations (e.g., to increase nutritional value), using nanoparticles (PS micelles) with hydrophilic shells. In this particular case, the polysorbate in the nanomicelles is not a natural component of fruit juice, simplifying the analytical strategy.

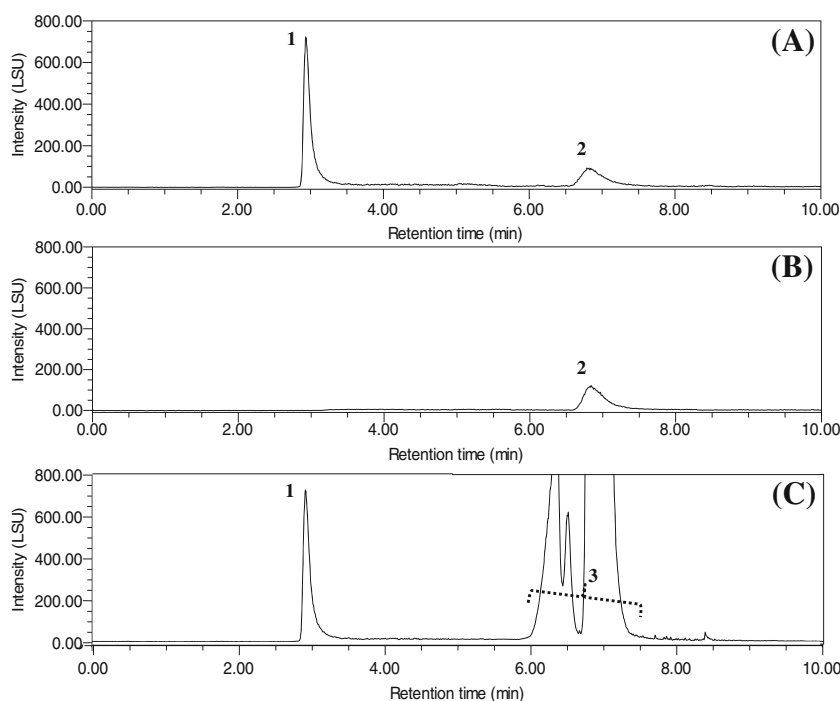
#### UHPLC-SEC-ELSD

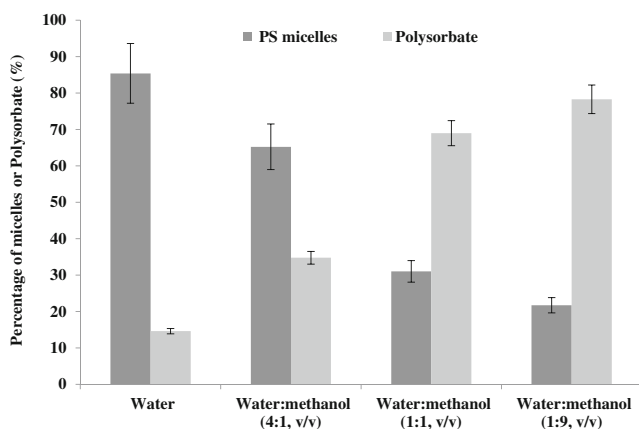
In contrast to inorganic NPs, the stability of organic NPs is a critical analytical issue. An ELS detector was employed in preliminary experiments, since it generally enables the detection of a broad range of chemical substances, including those without chromophores, such as polysorbates. When using reversed-phase liquid chromatography (RPLC) for the analysis of (i) aqueous solutions of polysorbate-based micelles and (ii) the respective polysorbate standards, similar chromatographic profiles were obtained; see Fig S2 of the ESM. Based on this information, we assumed that the PS micelles disassembled due to their interaction with the hydrophobic stationary phase and due to their instability in the presence of the organic solvent in the mobile phase. To remove these unfavorable factors, size-exclusion chromatography (SEC)

with an aqueous buffer solution as the mobile phase was considered a more feasible alternative for analyzing native PS micelles. For this purpose, an ACQUITY UPLC® BEH200 SEC column (designed for the fast separation of proteins with molecular weights in the range 10,000–450,000 Da) was employed. When isocratic elution with aqueous 5 mM ammonium formate (pH 5–6) was used to separate aqueous PS micelles, two peaks with retention times of 3.5 min and 6.6 min were detected (Fig. 2a). The latter Rt value was identical to that of the polysorbate 20/80 standard analyzed on the SEC column under the same conditions (Fig. 2b). This result confirmed (indirectly) that the first peak was due to PS micelles that eluted from the SEC column (in line with expectation), because a high effective volume was achieved fairly early compared to the free polysorbate. When the apple juice sample with added PS micelles was analyzed, the elution zone of the free polysorbate (partially present in the PS micelle solution) overlapped with that of the abundant low molecular weight matrix. Nevertheless, no interferences were detected close to the elution zone of the micelles; see Fig. 2c. This means that PS micelle quantification in apple juice is possible, assuming that a well-characterized reference standard is available. A linear increase in the PS micelle signal in apple juice was observed for concentrations in the range of  $0.5 \text{ mg mL}^{-1}$  to  $10 \text{ mg mL}^{-1}$ . The limit of detection (LoD) for the experimental PS micelles was  $0.5 \text{ mg mL}^{-1}$ .

Implementating the UHPLC-SEC-ELSD system enabled us to document micelle instability in the presence of a water-miscible organic solvent (mentioned above). As shown in Fig. 3, increasing the amount of methanol in solution resulted

**Fig. 2** UHPLC-SEC-ELSD analysis of aqueous solutions of **A** PS 20 micelles, **B** polysorbate 20 standard, **C** apple juice with added PS 20 micelles. Concentrations of the respective components in all samples  $\sim 10 \text{ mg mL}^{-1}$  (1: micelles; 2: polysorbate; 3: matrix components)





**Fig. 3** Stability of micelles in aqueous solutions depending upon amount of organic solvent added (measured max. 3 min after preparation)

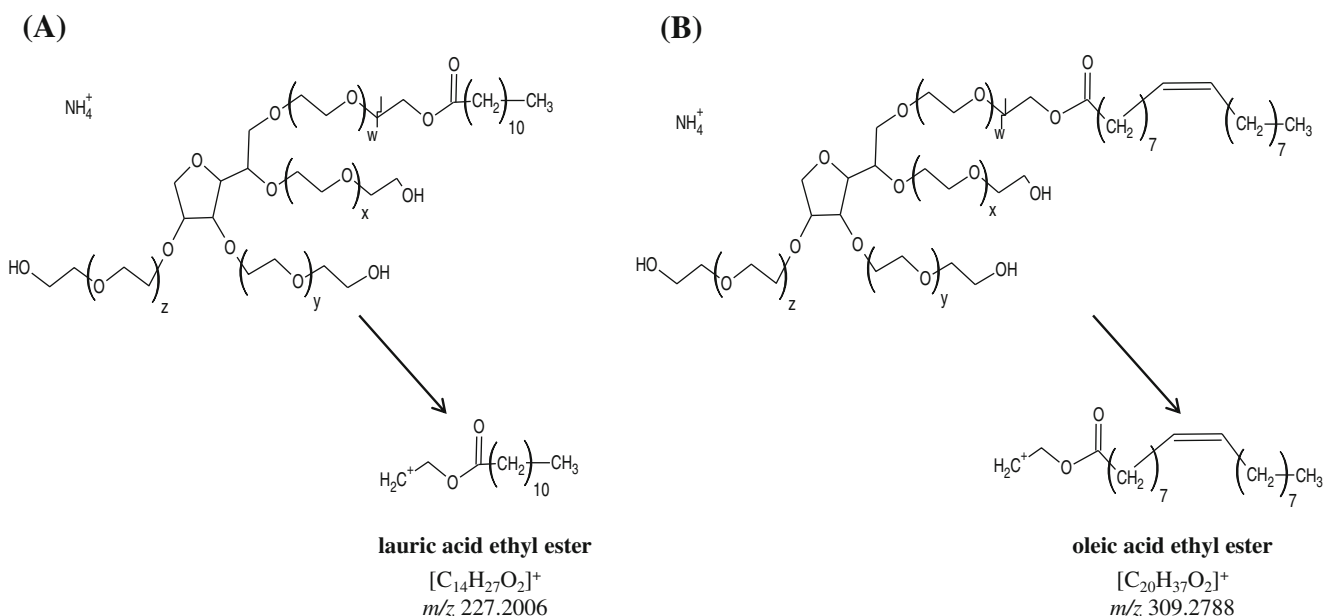
in PS micelle disintegration. Increasing the content of organic solvent resulted in a decrease in the height of the peak at the shorter retention time, while the height of the peak with the longer retention time, corresponding to free polysorbate released from micelles, increased. This technique provides a useful tool for verifying the presence of polysorbate micelles in fruit juice.

#### DART-Orbitrap MS

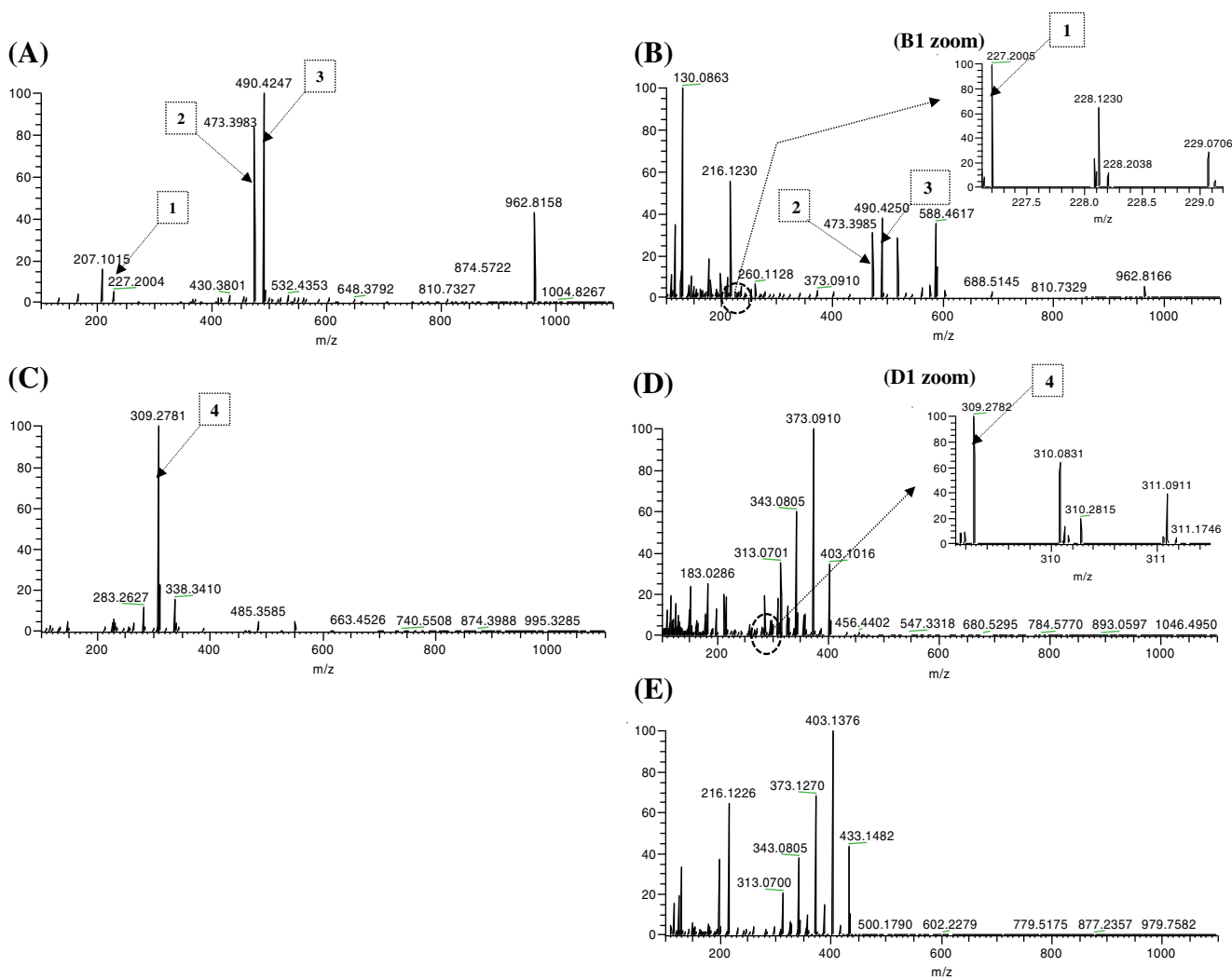
In the next phase, the potential of ambient mass spectrometry to rapidly detect polysorbate 20/80 was investigated. A direct analysis in real time (DART) ion source coupled with a high-resolution mass spectrometer with an Orbitrap mass analyzer was employed for this purpose. Specific positive fragment ions of lauric acid ethyl ester ( $m/z$  227.2006) and oleic acid

ethyl ester ( $m/z$  309.2788) originating from polysorbate and/or polysorbate micelles (Fig. 4) were present in the DART-HRMS positive spectra obtained from an aqueous PS micelle solution. Moreover, an intense ion signal from tocopheryl acetate ( $m/z$  473.3995), the active ingredient one of the tested PS micelles, was also present in the spectrum. The spectra of polysorbate 20/80 micelles in aqueous solution and apple juice are presented in Fig. 5. The lowest calibrated levels (LCL) for the PS micelles using DART-HRMS was  $0.5 \text{ mg mL}^{-1}$  and  $1 \text{ mg mL}^{-1}$  in water and apple juice, respectively.

In order to facilitate the rapid screening of PS micelles in apple juice, the DART-HRMS technique was validated at two concentration levels. Its performance characteristics are shown in Table 1. The concentrations of polysorbate micelles, 5 and  $10 \text{ mg mL}^{-1}$ , used to validate the DART-HRMS technique employed for the rapid screening of polysorbate in beverages were chosen based on information obtained from the NanoLyse project partner: the concentrations of nanoparticles added to beverages may range from 0.5 to 2 %. The measurement repeatability, expressed as the relative standard deviation (RSD), was around 23 % for a concentration level of  $10 \text{ mg mL}^{-1}$ , but rather higher for a concentration level of  $5 \text{ mg mL}^{-1}$ , 43 %. The repeatability could be improved to some degree by replacing the Dip-it sampling scanner employed in this study with an XY transmission module (which was not available in the laboratory during our experiments), as this allows for more controlled sampling of liquids. The heated metal screen enables more uniform thermal desorption of larger sample spots, thus leading to more accurate quantitative analyses than possible with impermeable sample



**Fig. 4** In-source fragments of **A** polysorbate 20 and **B** polysorbate 80



**Fig. 5** DART-HRMS positive spectra of **A** aqueous solution of PS 20 micelles, **B** apple juice with added PS 20 micelles, **C** aqueous solution of PS 80 micelles, **D** apple juice with added PS 80 micelles, **E** apple juice (blank). Concentrations of the respective components in all samples

$\sim 10 \text{ mg mL}^{-1}$  (**1**: lauric acid ethyl ester  $[\text{M}]^+$ ,  $m/z$  227.2006; **2**: tocopheryl acetate  $[\text{M}+\text{H}]^+$ ,  $m/z$  473.3995; **3**: ammonium adduct of tocopheryl acetate  $[\text{M}+\text{NH}_4]^+$ ,  $m/z$  490.4255; **4**: oleic acid ethyl ester  $[\text{M}]^+$ ,  $m/z$  309.2788)

holders. In any case, the use of an internal standard is recommended when trying to estimate analyte content. In this study, caffeine, which provides intense and stable positive ions (similar to polysorbate) was used to compensate for total ion current fluctuations during the repeated DART-MS measurements [33–35]. When caffeine is already present in the examined sample, another internal standard should be used. In general terms, isotope-labeled analyte analogues are the best option; however, in this particular case, labeled polysorbate was not commercially available.

#### UHPLC-HRTOF-MS

The analysis of PS micelles using the RPLC-ELSD system (Fig. S2 of the ESM) yielded a number of peaks for both the (disassembled) PS micelle sample and the

polysorbate standard. The UHPLC-HRTOF-MS technique was employed to study these compounds in detail. Complex chromatograms were obtained, with polyethylene glycols and polyethylene sorbitans eluting at shorter retention times. A wide range of polysorbate synthesis precursors together with polysorbate degradation products (e.g., dehydropolysorbates) and di-, tri-, and tetra-esterified polyoxyethylene sorbitans were tentatively identified. However, due to the presence of polysorbates with various numbers of ethoxylate units, yielding ions with different charge states (up to  $z=3$ ), the mass spectra were very complex and the number of ions detected was very high. Under these conditions, the ion mobility (IM)—a third resolution dimension—was employed. Using this method, peak capacity was increased and the clarity of the detected spectra used for identification was notably improved. Chromatograms and drift times

**Table 1** Recovery and repeatability of the DART-Orbitrap MS analytical method employed for the determination of PS micelles in apple juice

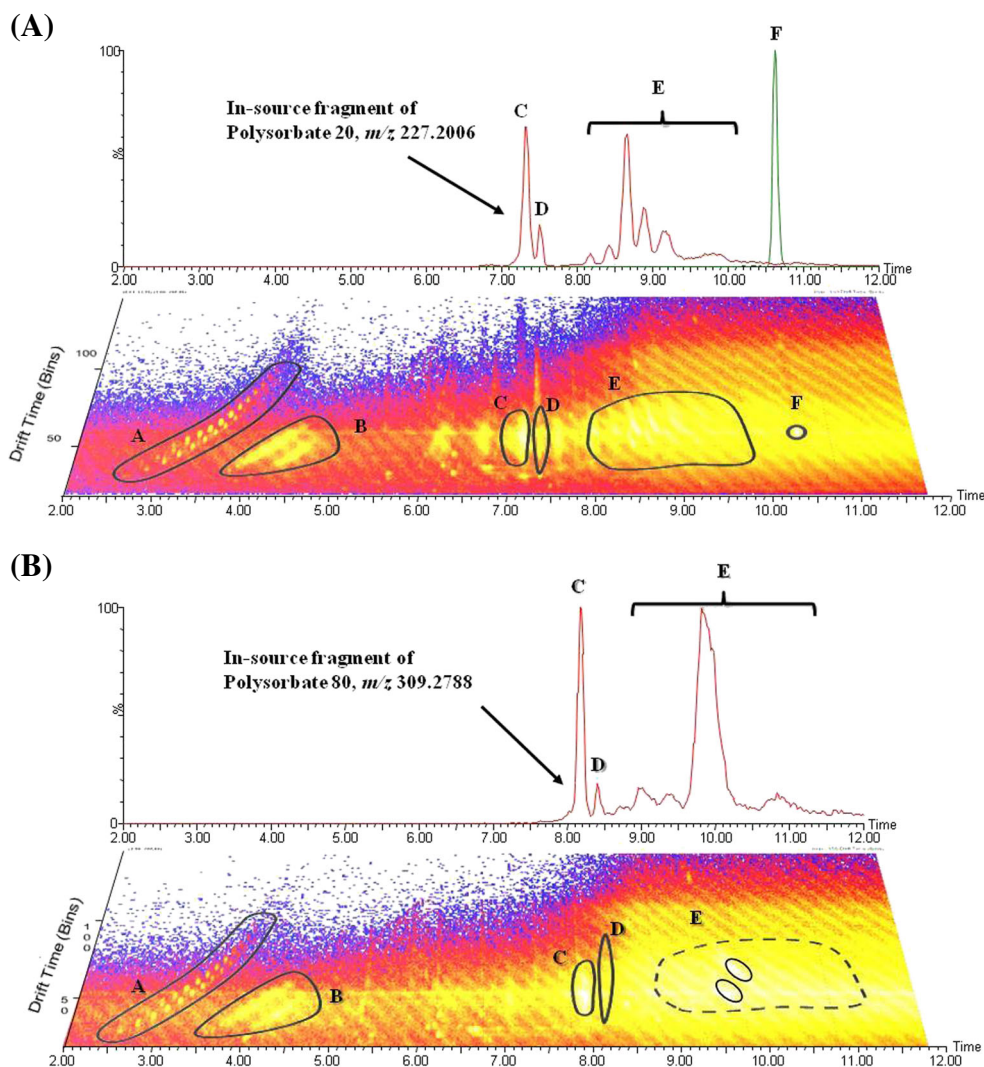
Spike ( $n=6$ )	Polysorbate 20 micelles ( $m/z$ 227.2006)		Polysorbate 80 micelles ( $m/z$ 309.2788)	
	Recovery range / average recovery (%)	RSD (%)	Recovery range / average recovery (%)	RSD (%)
10 mg mL <sup>-1</sup>	60–151 / 105	21	55–125 / 90	25
5 mg mL <sup>-1</sup>	56–191 / 124	47	51–180 / 115	45

for polysorbate 20 and 80 micelles are presented in Fig. 6, and these illustrate the complexity of the examined mixtures. Since, as mentioned above, the PS micelles disassembled immediately after dissolving in an aqueous medium containing organic solvents, the polysorbate 20/80 and polysorbate 20/80 micelles showed very similar chromatographic profiles.

Identification of the type of polysorbate present and its quantification were achieved by detecting specific in-source fragments (Fig. 4). Due to the rapid decomposition of PS micelles after dilution and during

chromatography (the forces between the polysorbate molecules that form the micelles are weak), commercially available polysorbate 20 and/or 80 standards could be used to prepare matrix-matched calibration solutions. The lowest calibrated level (LCL) of polysorbate specific in-source fragments ( $m/z$  227.2006 for polysorbate 20 and  $m/z$  309.2788 for polysorbate 80) is 0.5  $\mu\text{g mL}^{-1}$  in apple juice. Using these characteristic in-source fragments, the calibration curve for the analyzed standards was linear in the polysorbate concentration range of 0.5  $\mu\text{g mL}^{-1}$  to 20  $\mu\text{g mL}^{-1}$  (Fig. S3 of

**Fig. 6** UHPLC-IMS-TOF MS analysis of **A** PS 20 micelles and tocopheryl acetate as the active component, and **B** PS 80 micelles and glucose as the active component. Concentration 10  $\mu\text{g mL}^{-1}$  in apple juice. *A* polyethylene glycol, *B* polyoxyethylene sorbitan, *C* polysorbate 20/80, *D* dehydro polysorbate 20/80, *E* di-, tri-, and tetra esters of polysorbate 20/80 and their dehydro forms, *F* tocopheryl acetate)





**Table 2** Recovery and repeatability of the UHPLC-HRTOF-MS analytical method for determining PS micelles in apple juice

Spike ( $n=6$ )	Polysorbate 20 micelles ( $m/z$ 227.2006)		Polysorbate 80 micelles ( $m/z$ 309.2788)	
	Recovery range / average recovery (%)	RSD (%)	Recovery range / average recovery (%)	RSD (%)
10 $\mu\text{g mL}^{-1}$	98–103 / 100	2	92–100 / 96	3
5 $\mu\text{g mL}^{-1}$	96–108 / 102	3	98–103 / 100	5
1 $\mu\text{g mL}^{-1}$	92–115 / 105	19	90–120 / 110	21
Linear regression equation	$y=105.66x+53.324$		$y=119.99x+77.476$	
Regression coefficient ( $R^2$ )	0.9988		0.9979	

the ESM). Based on the results obtained, the total concentration of polysorbate used in PS micelles was in the range 80–95 %, depending on the type of PS micelle present. The recovery and repeatability of the UPLC-HRTOF-MS analytical method achieved when determining PS micelles during the validation study on apple juice are summarized in Table 2.

The LCL of tocopheryl acetate (encapsulated in NPs with vitamin E) was 0.1  $\mu\text{g mL}^{-1}$ . The detection was not specific for the other active compounds (glucose or medium-chain triglycerides)—these are commonly found to occur naturally in fruit juices.

## Conclusion

Currently, the use of polysorbate (PS) micelles in the food sector is being intensively investigated. Nanosized and/or nanoencapsulated ingredients intended for application in fruit juices represents one of the most challenging targets for analysis. Analytical strategies that can be applied to monitor the use of PS micelles in this context must be developed. The results obtained in the present study, which assessed various analytical methods for monitoring PS micelles, are summarized below.

The UHPLC-SEC-ELSD technique employing aqueous ammonium formate as a mobile phase can be used for the rapid (nonselective) screening of polysorbate micelles added to fruit juice. However, the type of polysorbate present in the micelle shells cannot be distinguished by this analytical strategy—micelles based on polysorbate 20 and micelles based on polysorbate 80 eluted at the same retention time. Also, free polysorbates co-elute with matrix components.

The DART-Orbitrap MS technique enables high-throughput screening and detection of polysorbate 20/80 (which are typically used in micelle shells) in apple juice. Some active PS micelle ingredients, such as tocopherol acetate, can be detected as well.

The UHPLC-HRTOF-MS technique represents a selective and sensitive analytical technique that can be employed for the

detection and quantification of polysorbate 20/80 (present in PS micelle building blocks) in apple juice. Moreover, this technique, when specifically coupled with IMS, enables comprehensive identification and detection of all of the impurities transferred into the fruit juice by the PS micelles.

The DART-Orbitrap MS and UHPLC-HRTOF-MS techniques were used to distinguish between polysorbate 20 and polysorbate 80 and to determine their levels in tested fruit samples, but it was not possible to discern whether the polysorbates were in the free or micellar form in the sample. Analysis of natural fruit juices (without added polysorbates and/or PS micelles) was performed, and those fruit juices did not contain polysorbates.

**Acknowledgments** The work leading to these results was funded by (i) the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 245162, *Nanoparticles in Food: Analytical Methods for Detection and Characterisation* (NanoLyse), (ii) the project MSMT no. 6046137305, and (iii) specific university research (MSMT no. 20/2014) supported by the Ministry of Education.

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