Title: Potential of supramolecular solvents for the extraction of contaminants in liquid foods

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Abstract

Amphiphile-based supramolecular solvents (ASSs), which are water immiscible liquids consisting of supramolecular aggregates in the nano and micro scale regimes dispersed in a continuous phase, were assessed for the extraction of trace contaminants in liquid foods. The ASS selected was made up of reversed micelles of decanoic dispersed in tetrahydrofuran (THF)-water and the contaminants used as a model were bisphenol A (BPA), ochratoxin A (OTA) and benzo(a)pyrene (BaPy). The influence of matrix components on the extractant solvent production, extraction recoveries and actual concentration factors was investigated by using commercial foods such as wine and wine-based products, beer, soft drinks and tea and coffee brews, and/or aqueous synthetic solutions containing specific food matrix components. The method involved the addition of decanoic acid (80 mg) and THF (0.8-1.7 mL) to the food sample (15 mL), stirring of the mixture for 5 min, centrifugation for 10 min and analysis of 10-20 μL of the extract by liquid chromatography coupled to fluorimetry for OTA and BaPy or to mass spectrometry for BPA. No clean-up of the crude extracts was required for any of the samples analysed. The quantification limits for the contaminants (14-31 ng L^{-1}, 0.37-0.39 ng L^{-1} and 562-602 ng L^{-1} for OTA, BaPy and BPA, respectively) were far below their respective European legislative threshold limits Recoveries for food samples were in the ranges 79-93%, 90-96 and 78- 82% for OTA, BaPy and BPA respectively, with relative standard deviations ranging from 1 to 7%, and actual concentrations factors between 65 and 141. The methods developed were applied to the determination of the target compounds in a variety of commercial foods. OTA was found in vinegar, must and beer samples, the concentrations ranging from 92 to 177 ng L^{-1}, BaPy was quantified in samples of tea and coffee at concentrations between 1.5 and 16.6 ng L^{-1} whereas BPA was detected in two canned soft drinks and quantified in one of them (tea beverage) at a level of 2.3 μg L^{-1}.

Keywords: Supramolecular solvents; Liquid-liquid extraction; Food analysis; Ochratoxin A; Bisphenol A; Benzo(a)Pyrene; Liquid chromatography; Mass spectrometry; Coacervates, Self-assembly

1. Introduction

Regulatory agencies and quality control laboratories are continuously demanding faster, simpler and cheaper methods for the analysis of trace contaminants in food. Sample preparation is nowadays the bottleneck in food analysis and there is a need to minimize the number of steps in order to reduce both time and sources of error [1]. Furthermore, methods must be sensitive enough to cover the decreasing legislative limits for food contaminants as well as more environmentally friendly.

Solvent extraction is by far the commonest technique used in official [2-4] and recently reported [5-7] methods for the extraction of contaminants from liquid foods, despite it often requires the use of large volumes of toxic organic solvents (typically 50-500 mL) and the subsequent evaporation and clean-up of the extracts. The amount of solvent required can be drastically reduced by using membrane-assisted extraction [8-10] or single-drop microextraction [11] however the suitability of these techniques for the extraction of trace contaminants is still in question because their efficiency is often matrix and analyte dependent [1]. On the other hand, regarding solid-phase extraction techniques, immunosorbents, although expensive and with limited liquid food applications so far, have become a good strategy for the extraction of ochratoxin A in wines [12] and phenylurea herbicides in fruit juices [13,14]. However, on the whole, there is still a strong requirement for more general and valuable sample preparation procedures that meet the demanding regulatory limits established [1].

This paper evaluates the capability of amphiphile-based supramolecular solvents (ASSs) to extract trace contaminants in liquid foods. The term *supramolecular solvent* is here introduced for the first time to design water-immiscible liquids made up of supramolecular assemblies dispersed in a continuous phase. ASSs are produced from amphiphile solutions by two well-defined self-assembly processes occurring on two scales, molecular and nano. First, amphiphilic molecules spontaneously form three-dimensional aggregates above a critical aggregation concentration, mainly aqueous (size 3-6 nm) and reversed micelles (size 4-8 nm), and vesicles (size 30-500 nm), depending on the structure of the amphiphile and solvent properties. Then, the generated nanostructures self-assemble in larger aggregates with a wide size distribution in the nano and micro scale regimes by the action of an external stimulus (e.g. temperature, electrolyte, pH, solvent) and separate from the bulk solution by a mechanism that remains elusive. The phenomenon of liquid-liquid phase separation, named *coacervation* [15], occurs in many colloidal solutions containing proteins, carbohydrates and polymers and it is widely used for microencapsulation of active ingredients in pharmaceuticals and food [16].

Supramolecular solvents are, by definition, incompatible with the solvent from which they originated despite this solvent is a major component of ASSs and constitutes the continuous phase in which the supramolecular assemblies disperse [17]. Likewise supramolecular solvents are reversible; the ordered structures assemble through non-covalent interactions and may dissasemble in response to environmental factors or external stimuli, so ASSs behave as adaptive materials [18]. Figure 1 shows a typical micrograph of an ASS and depicts the common nanostructures that make it up.

The outstanding properties of ASSs for extraction processes derive from the special structure and high concentration of the ordered aggregates that constitute them. Supramolecular assemblies have regions of different polarity that provide a variety of interactions for analytes. The type of interaction may be tuned varying the hydrophobic or the polar group of the amphiphile and in theory we may design the most appropriate ASS for a specific application because amphiphiles are ubiquitous in nature and synthetic chemistry. A major feature of ASSs is the high concentration of

amphiphiles, and therefore of binding sites, they contain (typically 0.1-1 mg μL^{-1}). Consequently, high extraction efficiencies can be achieved using low ASS volumes which results in high concentration factors (typically 100-500).

Non-ionic micelle-based supramolecular solvents have been applied to the extraction of contaminants in environmental aqueous samples for a long time and the corresponding extraction approach has been named *cloud point technique* in the analytical literature [19-21]. The development of supramolecular solvents based on zwitterionic [22], cationic [23] and anionic [24] micelles avoided the problems of coelution caused by non-ionic surfactants in LC and made compatible ASSs with MS, which permitted their application to the extraction of pollutants from sludge and soils [25,26]. Recently, ASSs made up of vesicles [27] and reversed micelles [28] of alkyl carboxylic acids have been reported and have marked a turning point with regard to the type of aggregates that constitute them, the variety of interactions they can establish with analytes and the high concentration of amphiphiles they contain.

This paper explores for the first time the suitability of ASSs for the development of simple, robust and reliable sample preparation methods for the determination of contaminants in liquid foods. For this purpose, bisphenol A (an endocrine disrupter migrating from food packaging materials), ochratoxin A (a carcinogenic mycotoxin) and benzo(a)pyrene (a carcinogenic polycyclic aromatic hydrocarbon produced in food processing) were selected as model analytes. The food matrices investigated included wine, vinegar, must, beer, soft drinks and tea and coffee brews. The supramolecular solvent made up of decanoic acid reversed micelles was used as extractant [28]. The selection of this ASS was based on its capacity to bind analytes through hydrophobic and hydrogen bond interactions and its low volume. Liquid chromatography coupled to fluorescence or mass spectrometry was used for the quantitation of the extracted contaminants. The influence of food matrix components on the formation and behavior of the selected ASS was investigated, the parameters affecting extraction efficiencies and concentration factors were optimized, the analytical characteristics of the developed methods were established and they were successfully applied to the determination of contaminants in a variety of liquid foods.

2. Experimental

2.1. Chemicals

All chemicals were of analytical reagent-grade and were used as supplied. Decanoic acid (capric) was obtained from Fluka (Madrid, Spain). Tetrahydrofuran (THF) and LC-grade acetronitrile were supplied by Panreac (Sevilla, Spain), and ultra-high-quality water was obtained with a Milli-Q water purification system (Millipore, Madrid, Spain). The target compound bisphenol A [BPA; 2,2′-bis(4-hydroxyphenyl)propane] was obtained from Fluka while benzo(a)pyrene (BaPy) and Ochratoxin A (OTA) were purchased in Sigma-Aldrich (St. Louis, MO, USA). The biomolecules sucrose, D-(-)-fructose and D-(+)-glucose were acquired from Sigma-Aldrich, and albumin from bovine serum, albumin from chicken egg white and lysozyme from chicken egg were obtained from Fluka. Stock standard solutions of 1 g L^{-1} of BPA in acetonitrile, 100 mg L^{-1} of BaPy in acetonitrile and 10 mg L^{-1} of OTA in methanol, were stored under dark conditions at -20ºC. Working solutions were made by appropriate dilutions of the stock solutions with acetonitrile or methanol.

200 *2.2. Apparatus*
201
202 The liquid chromatographic system used to quantify OTA and BaPy (Spectra System SCM1000,
203 ThermoQuest, San Jose, CA, USA) consisted of a P2000 binary pump and a FL3000 fluorescence
204 detector (LC-FL). In all experiments a PEEK Rheodyne 7125NS injection valve with a 20 µL
205 sample loop was used (ThermoQuest, San Jose, CA, USA). The stationary-phase column was a
206 Hypersil ODS C_8 (5 µm, 150 x 4.6 mm) from Analisis Vínicos (Tomelloso, Spain). Quantitation of
207 BPA was made using a liquid chromatography/electrospray ion trap-mass spectrometry system
208 (LC/(ESI-IT)-MS) (1100 Series LC/MSD, Agilent Technologies, Waldbronn, Germany) equipped
209 with an automatic injector (injection volume 10µL). The stationary-phase column was a Hypersil
210 ODS C_8 (3 µm 50 x 2.1 mm) from Analisis Vínicos (Tomelloso, Spain). A Mixtasel Selecta
211 centrifuge (Barcelona, Spain) was used for sample preparation.
212
213 *2.3. Determination of BPA, BaPy and OTA in liquid foods*
214
215 *2.3.1. Sample collection and preparation*
216 Liquid foods (n=19) were purchased in different supermarkets in Córdoba (Spain) and their content
217 for BPA (canned tea and lemon soft drinks and canned white soda), BaPy (red and mate tea and
218 soluble coffee brews) and OTA (must, vinegar, white and red wine and beer) was investigated.
219 Sealed samples were stored at room temperature until analysis. Before extraction, carbonated soft
220 drinks and beer samples were degassed in an ultrasonic bath for 30 min. Beer was then diluted with
221 distilled water (1:1, v/v). Soluble coffee and tea brews were prepared according to the instructions
222 on the product label. Thus, coffee (2g) was dissolved in 250 mL of boiling distilled water and tea
223 brew was obtained by boiling 3.5 g of sample in 500 mL of distilled water in an enclosed steel
224 kettle for 15 min. After cooling at room temperature, tea brews were filtered with an ashless filter
225 paper and made up to 500 mL with distilled water.
226
227 *2.3.2. Supramolecular solvent-based extraction*
228 Decanoic acid (80 mg) was dissolved in THF (0.8mL for the analysis of OTA and BaPy and 1.7mL
229 for the analysis of BPA) into specially designed centrifuge tubes (Figure 2). Then, the liquid food
230 (15 mL; pH ~2-3.6, adjusted when necessary with HCl 12M) were added. Immediately, the
231 supramolecular solvent spontaneously formed into the bulk solution. The mixture was stirred (5
232 min, 700 rpm) to favour BPA, BaPy and OTA extraction and then centrifuged at 3800 rpm (2200 g)
233 for 10 min to accelerate the separation of the supramolecular solvent from the bulk solution. Beer
234 samples required a centrifugation time of 30 min to achieve good separation. Finally, the volume of
235 supramolecular solvent (typically 100-150 µL), which was standing at the narrow neck of the
236 centrifuge tube (Figure 2), was calculated by measuring its height with a digital callipers, and 10 or
237 20 µL were withdrawn with a microsyringe and directly injected into the LC-MS or the LC-FL
238 system, respectively.
239
240 *2.3.3. Quantitation of BPA, BaPy and OTA*
241 Quantitation of BPA was carried out using LC/(ESI-IT)-MS. The mobile phase consisted of
242 acetonitrile and water (60:40, v/v) at a flow rate of 0.2 mL min^{-1} for 15 min. The diver valve was
243 programmed to send the mobile phase containing carboxylic acid and the most polar matrix
244 compounds to waste. So, only 6 min after the beginning of the elution gradient program, the eluted
245 components were sent to the ionisation source. Mass spectrometric analysis of BPA was performed
246 in the ESI(-) mode. The molecular ion (*m/z* 227) was isolated and fragmented into the ion trap.
247 Excitation of the ion was accomplished through collision with helium. The set of the parameters
248 used was as follow: capillary voltage, 5.0 kV; capillary exit voltage, -165 V; skimmer, -44 V; trap
249 drive, -31, source temperature, 350 ºC; drying gas, 5 L min^{-1}; nebulizer gas, 80 psi; maximal

accumulation time, 100 ms, resonance excitation 1.12 V and fragmentation time 100 ms. Quantification was carried out under full scan (200-250 *m/z*) by monitoring the extracted ion chromatogram at the *m/z* of the daughter ion 212 $[M\text{-}H\text{-}CH_3]^-$. Calibration curves were performed in acetonitrile and were linear from 50 to 1000 μg L^{-1}.

Quantification of BaPy was carried out by LC-FL by measuring peak areas at 284 and 404 nm (excitation and emission wavelengths, respectively). The mobile phase consisted of water (solvent A) and acetonitrile (solvent B) at a flow rate of 1mL min^{-1}. The gradient elution program was: 25% A for 5 min and from 75% to 100% B in the next 20 min. Calibration curves in acetonitrile were linear in the range 0.05-500 μg L^{-1}.

LC-FL was also used for quantitation of OTA. The mobile phase consisted of water (solvent A) and acetonitrile (solvent B), both containing 1% acetic acid. The gradient elution program was linear from 60% to 50% in A for 15 min and then isocratic with 50% A during 10 min. The flow-rate was 1 mL min^{-1}. OTA was monitored at 334 nm and 460 nm of excitation and emission wavelengths. Calibration curves for OTA in methanol were linear in the range 2-5000 μg L^{-1}.

3. Results and discussion

3.1. Supramolecular solvent description

Decanoic acid dissolves in THF forming reverse micelles according to a sequential-type self-association model [29] with at least three critical aggregation concentrations (4.8±0.2, 7.6±0.4 and 51±2 mM) [28]. The addition of water to this binary system causes partial desolvation of the aggregates, which makes their interaction easier and promotes the formation of larger reverse micelles that separate from the THF:water bulk solution as an immiscible liquid phase. So, water is the external stimulus that causes the coacervation. The resulting supramolecular solvent consists of reverse micelles with a wide size distribution in the nano and micro scale regimes, dispersed in a THF:water continuous phase. As reverse micelles are produced from the protonated decanoic acid form (pK_a 4.8±0.2), pH values below 4 are required for the formation of the supramolecular solvent.

Figure 3A shows the relative concentration of the three supramolecular solvent components at which the coacervation occurs and consequently the supramolecular solvent is produced. Beyond the boundaries of this region, the decanoic acid precipitates or solubilizes in the THF:water bulk solution. The reversed micelles in the supramolecular solvent provide a 2-fold mechanism for analyte solubilisation, namely van der Waals interactions in the decanoic acid hydrocarbon chains and hydrogen bonds in the micellar core, so a number of analytes can be extracted efficiently from aqueous samples with this solvent. Table 1 shows the chemical structures of the contaminants selected in this study and the constants and data of interest for their extraction.

3.2. Formation of the supramolecular solvent in liquid foods

Liquid foods have high water content [30], so they were expected to induce the coacervation of reverse micelles of decanoic acid. Production of the ASS was investigated in ternary systems consisting of decanoic acid, THF and a variety of liquid foods, which were selected to cover a wide range of matrix composition. Table 2 reports the concentration of major matrix components in the liquid foods investigated [30,31] and Figure 3B depicts the phase diagrams obtained for some representative foods. Like water, all the samples induced the coacervation of decanoic acid and the phase diagrams showed three regions where the decanoic acid was precipitated, coacervated or solubilized. So, this supramolecular solvent is suitable to extract contaminants from liquid foods.

According to the effect of food matrix components on the upper boundary in the phase diagram compared with that obtained in distilled water, liquid foods may be classified in three groups. Foods belonging to group I (vinegar, red and mate tea and coffee brews; e.g. Fig 3B, line 2) behaved similarly to water; those belonging to group II (beer and red and white wine; e.g. Fig.3B, lines 3 and 5) moved the upper boundary in the phase diagram toward lower THF percentages; and those belonging to group III (tea and lemon soft drinks, white soda and must; e.g. Fig.3B, lines 1 and 4) exerted a double effect, first they increased the THF percentage required to dissolve the supramolecular aggregates, and second they caused the separation of THF and the liquid food into two immiscible phases as the concentration of THF was above a limit [around 65% for must (line 4) and 90% for soft drinks (line 1)]. On the other hand, whitish and reddish precipitates, which were standing at the bottom of the supramolecular solvent as a very thin layer were extracted in beer and red wine samples, respectively.

In order to explain the observed phenomena and establish the basis for the prediction of phase diagrams as a function of food matrix components, a working hypothesis was established on the basis of the food compositions causing them (cf. Table 2). This hypothesis was as follows: above an unknown concentration, ethanol decreases the coacervating region; sugar increases the coacervating region and makes immiscible THF and liquid foods; and proteins and condensed tannins flocculate in the medium required to produce the supramolecular solvent. To support the correctness of this hypothesis, phase diagrams of ternary mixtures consisting of decanoic acid, THF and synthetic aqueous solutions containing matrix components at different concentrations were constructed.

Figure 4A shows some of the phase diagrams obtained for ethanolic aqueous solutions containing ethanol concentrations up to 15%. The upper boundary in the phase diagram moved towards lower THF percentages compared to aqueous solutions for ethanol percentages above 3% thus confirming the results obtained for beer and wine samples (cf. Fig.3B, lines 3 and 5), which have ethanol contents in the ranges 3-5% and 10-13%, respectively (cf. Table 2). The effect of ethanol was expected on the basis that the transition from the coacervation to the isotropic solution region occurs by dissolution of the supramolecular aggregates in the organic solvent. Consequently, if a liquid food contains ethanol, lower THF amount will be necessary to dissolve the aggregates.

As all the foods included in group II contained a high sugar concentration (e.g. tea and lemon soft drinks, white soda and must, cf. Table 2), phase diagrams were constructed for sugary aqueous solutions containing a mixture of sucrose, glucose and fructose, each at the same concentration, at levels varying between 0 and 250 mg mL^{-1}. Figure 4B shows some of the results obtained. Sugar concentrations below ~90 mg mL^{-1} did not affect phase diagrams (Fig.4B, line 1). Above this concentration (Fig.4B, lines 2-4) sugary aqueous solutions behaved similarly to the foods included in group II (cf. Fig. 3B, lines 1 and 4). Thus, sugar increased the THF percentage required to dissolve the coacervate in an isotropic solution (curve portion of lines 2-4 in Fig.4B) and caused THF:water phase separation (linear portion of lines 2-4 in Fig.4B). The THF percentage at which this solvent and water were immiscible decreased as the sugar concentration increased and it was independent of the decanoic acid concentration. Thus, THF:water phase separation was also known to occur in binary systems made up of THF and sugary aqueous solutions (see broken lines in Fig. 4B). To our knowledge, no information about this phenomenon has been previously reported. However, it seems to be related to a salting out effect [32] due to the fact that sugars decrease water activity by producing a statistically reduced number of available hydrogen bonding sites, the effect

346 increasing with sugar concentration [33]. We checked that the rough limit found for sugary aqueous
347 solutions (~90 mg mL^{-1}) from which sugar started exerting effect on phase diagrams was applicable
348 to a range of commercial liquid foods. Thus, apple (sugar: 129.3 mg mL^{-1}) and orange (sugar: 98.5
349 mg mL^{-1}) juices affected the phase diagram and a cola low calorie soft drink (no sugar) did not
350 affect it at all.

351

352 From an analytical point of view, it is worth noting that the effect of sugar and ethanol on phase
353 irrelevant to the use of the supramolecular solvent in extractions since analytical applications are
354 usually carried out near the lower phase boundary in order to use the minimal amount of THF [28,
355 34].

356

357 To check whether the white precipitate observed in beer samples was caused by proteins, the effect
358 of THF and decanoic acid reverse micelles on aqueous solutions containing a mixture of albumin
359 from bovine serum, albumin from chicken egg white and lysozyme, each at the same concentration,
360 at levels varying between 0 and 10 mg mL^{-1}, was investigated. The results showed that proteins
361 remained as stable colloids under the addition of THF, however they flocculated in the presence of
362 decanoic acid reverse micelles and were extracted by the supramolecular solvent, from which
363 separated after centrifugation as a thin layer at the bottom. This layer became wider as the protein
364 concentration increased and was clearly detectable for proteins concentrations above 1 mg mL^{-1},
365 which agrees with the results obtained for beer samples (protein concentration 4.48 mg mL^{-1}, Table
366 2). On the other hand, the precipitate became denser and so the layer narrower as the THF
367 concentration increased. It was checked that other liquid foods containing high protein
368 concentration (e.g. orange juice, 5.90 mg mL^{-1}) behaved similarly. The extraction of proteins by
369 reverse micelles has previously been proposed in the literature [35] and constitutes a valuable
370 method for their purification. On the other hand, regarding the reddish precipitate observed in red
371 wine samples, condensed tannins have been reported to bond to proteins and form large colloidal
372 particles, being the most frequent cause of hazes in these beverages.[36]. So, these macromolecules
373 were probably flocculated by the reverse micelles and extracted by the supramolecular solvent.
374 Below, the influence of theses precipitates on the extraction of the target analytes will be
375 investigated.

376

377 *3.3. Optimisation of the supramolecular solvent-based extraction*

378

379 Optimisation was carried out by extracting distilled water and liquid foods (15 mL) fortified with 1
380 mg L^{-1} of OTA (wine, vinegar, must and beer), 20 mg L^{-1} of BPA (white soda) and 0.2 mg L^{-1} of
381 BaPy (tea and coffee brews) under a variety of experimental conditions (0.1–3% decanoic acid; 5-
382 15% THF; pH 0.5-3.6; stirring time 0-20 min). Experiments were made in triplicate. Selection of
383 the optimal conditions was based on the recoveries (R) and actual concentration factors (ACF)
384 obtained for the target compounds. Phase volume ratios (PVR) were calculated as the ratio of the
385 sample volume over the supramolecular solvent volume, so they represented the maximum
386 concentration factors that could be obtained under given experimental conditions.
387
388
389 *3.3.1. Phase volumes ratio*
390 The volume of supramolecular solvent produced in water samples has previously been known to
391 mainly depend on the concentration of decanoic acid and THF, which are major components of this
392 solvent [34]. Water hardly incorporates to the extractant phase due to its non-solvent character for

the reverse micelles. General equations have been developed for the prediction of the volume of ASS produced in water solutions [34], *y,* as a function of the amount of decanoic acid ($y = a + bx$), the percentage of THF ($y = b_0 e^{b_1 z}$), and both the amount of decanoic acid and percentage of THF ($y = 1.035\ x\ e^{0.04731z}$). In these equations, *y* is given in μL, *x* is the amount of decanoic acid in mg, and *z* the THF percentage (v/v).

In order to determine whether food matrix components influenced the volume of supramolecular solvent produced, a set of experiments was carried out using different decanoic acid amounts (50-500 mg), THF concentrations (2.5-30%) and liquid foods (cf. Table 3). The volumes of ASS obtained were measured with a digital calliper and the relationship between these volumes and the amount of decanoic acid and THF was investigated. Table 3 shows the results obtained. Data in distilled water are also included for comparison.

As expected, the volume of coacervate was linearly dependent on the amount of surfactant used. This type of dependence indicates that the composition of the supramolecular solvent keeps constant when the other variables remain unchanged. The slopes of these linear relationships ranged between 1.60 and 1.86 μL mg^{-1}. The highest values were obtained for wine (1.86 μL mg^{-1}) and beer (1.79 μL mg^{-1}) thus indicating that some proportion of the ethanol content in this samples incorporated to the coacervate. The rest of matrix components did not influence the volume of the supramolecular solvent. Thus, the mean value for the slope in liquid foods, excluding wine and beer, (1.66±0.04 μL mg^{-1}) indicated that the composition of the supramolecular solvent was similar to that produced in water (1.67 μL mg^{-1}).

The relationship between the volume of supramolecular solvent and the THF percentage was exponential for all the foods investigated (see Table 3). The parameter (b_1), which describes how rapidly the volume of coacervate increases as the THF (%) does [34], was found to be similar in all the experiments (mean value 0.046±0.001), thus indicating that it was not influenced by matrix components. On the contrary, the parameter b_0, which is linearly related to the amount of decanoic acid [34], increased for ethanol-containing foods (e.g. beer and wines in Table 3). No significant differences were found for b_0 from the rest of liquid foods (mean 204±3 μL) and water (205 μL).

According to these results, the highest phase volume ratios will be obtained using low amounts of decanoic acid and THF. So, coacervate compositions near the lower boundary in the phase diagrams (Figure3B) are recommended for extraction.

3.3.2. Recoveries and actual concentration factors)

The influence of variables on recoveries (R) was studied and the actual concentration factors, ACF [0.01R(%) x phase volume ratio (PVR)], were calculated from the volumes of supramolecular solvent predicted by the respective equations (cf. Table 3). Tables 4 and 5 show the results obtained for the different foods investigated as a function of decanoic acid and THF concentration, respectively. Data in distilled water were also included in order to evaluate the effect of matrix components on analyte recoveries and consequently on ACF.

Matrix-dependent recoveries were found at the lowest decanoic acid concentration investigated (0.1% in Table 4). The recoveries increased as the amount of decanoic did and it was above 79% and matrix-independent, except for OTA in beer, at a decanoic acid concentration as low as 0.5%. The effect observed in beer was due to the adsorption of OTA in the flocculated protein layer standing at the bottom of the ASS. The adsorption decreased progressively as the decanoic acid increased and became negligible at concentrations above 2%, due to the gradual increase in the

coacervate/protein layer volume ratio. Dilution of beer with water (1:1; v/v) permitted to overcome this matrix-effect and beer behaved as the rest of foods (Table 4). Contrarily, the precipitate caused by condensed tannins in red wines scarcely affected OTA recoveries despite it was standing at the bottom of the ASS after sample centrifugation too. On the other hand, ACF values decreased as the decanoic acid concentration did (because of the decrease in phase volume ratios predicted by the equations proposed in Table 3). A concentration of 0.5% was selected as optimal on the basis that it provided the best ACF for the target compounds at R values higher than the threshold value (70%) recommended by different international organisations for the extraction of contaminants [35-37].

The influence of THF (5-15%) on R depended on the type of analyte; recoveries hardly changed for OTA and BaPy and slightly increased for BPA as the THF concentration did up to 10% (Table 5). Except for no diluted beer, no significant matrix effects were observed at the different percentages of THF investigated. A percentage of 5% was selected for OTA and BaPy while a 10% THF was recommended for BPA. Recoveries higher than about 80% were obtained at these THF concentrations.

The pH of samples did not affect recoveries in the range 2-3.6, but it caused a slightly decreased at lower pHs (e.g. recovery of OTA in wines was 80% at pH 0.5). As soft drinks and wine-based products have pHs between 2 and 3.6, it was not necessary to adjust them before extraction, while the pH of tea and coffee brews (pH 6-7) and beer (pH 4-4.5) was adjusted to 2.5 to ensure that decanoic acid was protonated, which is a requisite to form the ASS. Extraction equilibrium conditions were rapidly reached; maximal recoveries for the three analytes in all the matrices investigated were achieved after stirring the samples for 5 min at 700 rpm.

A practical aspect to be considered was the volume of liquid food sample to analyse, because although it does not influence recoveries or concentration factors, it determines the total mass of decanoic acid at a given surfactant concentration and consequently the volume of coacervate obtained. Our criterion was to obtain at least 100 µL of supramolecular solvent per sample, which permitted 2-3 different chromatographic runs in a reliable way (20 µL per injection). So, a volume of liquid food of 15 mL (7.5 mL for beer and made up to 15mL with distilled water) was chosen, which provided volumes of supramolecular solvent between 100 and 150 µL.

3.4. Analytical performance

Calibration curves for the target compounds were run using standard solutions prepared in acetonitrile (BPA and BaPy) or methanol (OTA). No differences in peak areas or retention times were observed for the analytes injected in organic solvent or the supramolecular solvent. The retention times for analytes, linear ranges, slopes of the calibration curves and correlation coefficients are included in Table 6. The instrumental quantification and detection limits were calculated from blank determinations by using a signal-to-noise ratio of 10 and 3, respectively. From these values and considering the ACF obtained for the different foods investigated, under the optimal experimental conditions proposed in section *2.3.2*, a range of estimated method quantification and detection limits were calculated (Table 6). These values were far below the current threshold limits established by the European Union with regards to the target compounds in different foodstuffs, i.e. 2 mg Kg^{-1} for OTA in wine and wine derived products [43], 600 mg Kg^{-1} for BPA as specific migration limit [44], values between 1 and 10 mg Kg^{-1} for BaPy [45]. Currently, a recommended level of 0.2 µg L^{-1} has been proposed for beer [46].

The possible interference of matrix components that could elute with the analytes was assessed by comparison of the slopes of the calibration curves for each compound (n =7) obtained from standards in distilled water with those obtained from liquid foods, namely three samples for OTA (wine, vinegar and must), two samples for BaPy (soluble coffee and tea) and two samples for BPA (white soda and tea soft drink). Slopes in distilled water standards were 7.0±0.4 L mg^{-1} for BPA, 13.2±0.4 L mg^{-1} for OTA and 456±6 L mg^{-1} for BaPy, while in foods were in the ranges 6.9-7.2 L mg^{-1} for BPA, 11.7-13.8 L mg^{-1} for OTA and 420-442 L mg^{-1} for BaPy, with relative standard errors of the slopes between 2 and 6 %. Differences in both types of calibration curves were only due to the slightly different ACF values reached in the foods compared to water, so matrix components were not expected to interfere in the determination of the three target compounds.

The precision of the method for the determination of OTA, BaPy and BPA was assessed by the extraction of eleven independent fortified samples, which consisted of wines (n=4), musts (n=4) and vinegar (n=3) for OTA, red tea (n=3), mate tea (n=3) and soluble coffee (n=5) for BaPy and white soda (n=5) and tea (n=6) soft drinks for BPA. Values expressed as relative standard deviations were 5.1, 5.4 and 6.1% for OTA, BaPy and BPA, respectively.

3.5. Analysis of liquid foods

A variety of liquid foods were analysed belonging to different trademarks than those used for optimisation. Table 7 shows the concentrations found for the different target compounds as well as the recoveries obtained after spiking the samples with variable amounts of OTA, BaPy and BPA, which are specified in the table footnote. Both the concentrations of analytes and recoveries were expressed as the mean value of three independent determinations, besides their corresponding standard deviations. Recoveries ranged between 79 and 93%, 90 and 96 and 78 and 82% for OTA, BaPy and BPA respectively, with relative standard deviations ranging from 1 to 7%.

OTA was detected in vinegar, must and beer samples, the concentrations ranging between 92 and 177 ng L^{-1}, BaPy was quantified in samples of tea and coffee at concentrations from 1.5 to 16.6 ng L^{-1} (equivalent to 0.22 and 2.1 mg Kg^{-1}, respectively) and BPA was detected in two canned soft drinks and quantified in one of them (tea beverage) at a level of 2.3 μg L^{-1}. These values were far below the established European threshold limits, except for the concentration of BaPy found in the mate tea sample (2.1 mg Kg^{-1}).

Figures 5-7 compare the chromatograms obtained from standard solutions (A) with those obtained from the analysis of different non spiked foodstuffs contaminated with OTA (Fig. 5), BaPy (Fig. 6) and BPA (Fig. 7). No interference from matrix components was detected for any of the samples analysed.

4. Conclusions

The results obtained in this research prove that supramolecular solvents are a promising strategy to simplify sample treatment in liquid food analysis. Supramolecular solvents are produced *in situ* through self-assembly processes that are within everyone´s reach. Likewise, extraction procedures are simple and do not require special lab equipment. The high concentration of decanoic acid in the extractant phase (~0.6 mg μL^{-1}) and, mainly the capability of analyte solubilization of the nanostructures formed, permits the favourable partition of analytes using a quite low volume of supramolecular solvent (100-150 μL) for 15 mL of sample in the application here developed). Consequently, actual concentration factors around 65-141 are easily obtained using a single-step

extraction and without the need of solvent evaporation. A valuable asset of this strategy is that major matrix components in the liquid foods (e.g. proteins) are not dissolved in the extractrant phase; they flocculate and remains as a precipitate at the bottom of the supramolecular solvent. So, crude extracts can be directly injected in the chromatographic system.

In this research, methods have been developed that permit the determination of OTA, BPA and BaPy in liquid foods at levels far below their respective European legislative threshold limits with recoveries higher than 80% and RSD values below 7%. Each complete extraction procedure took about 15-20 min and several samples could be simultaneously extracted, so sample throughput will mainly depend on the chromatographic analysis of the target compounds.

Acknowledgments

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637
638 **Figure captions**
639
640 **Figure 1.** Light microscopy (bright field) micrograph of a typical amphiphile-based supramolecular
641 solvent, and schematic picture of the aggregates that may constitute it.
642
643 **Figure 2.** Schematic picture of the glass centrifuge tube designed for ASS-based extractions.
644
645 **Figure 3.** Phase diagrams for decanoic acid in binary mixtures of (A) THF and water and (B) THF
646 and (1) lemon soft drink, (2) red tea infusion, (3) beer, (4) must and (5) wine.
647
648 **Figure 4.** Phase diagrams for decanoic acid in binary mixtures of: (A) THF and ethanolic aqueous
649 solutions containing (1) 2, (2) 4, (3) 8 and (4) 15% (v/v) of ethanol, and (B) THF and sugary
650 aqueous solutions containing a mixture of sucrose, glucose and fructose, each at the same
651 concentration, at an overall sugar concentration of (1) 50, (2) 115, (3) 150, (4) 180 and (5) 250 mg
652 mL^{-1}. The broken lines represent the boundaries for binary systems made up of THF and sugary
653 aqueous solution for an overall sugar concentration of (2)115, (3) 150, (4) 180 and (5) 250 mg mL^{-1}.
654 All the solutions were adjusted to pH 2.5.
655
656 **Figure 5.** LC/Fluorescence chromatograms obtained from (A) OTA (20 μg L^{-1}) in methanol and
657 two contaminated samples: (B) white wine must (177 ng L^{-1}) and (C) vinegar (92 ng L^{-1}).
658
659 **Figure 6.** LC/Fluorescence chromatograms obtained from (A) BaPy (10.5 μg L^{-1}) in acetonitrile
660 and two contaminated samples with BaPy, (B) mate tea brew (16.6 ng L^{-1}) and (C) instant coffee
661 brew (1.5 ng L^{-1}).
662
663 **Figure 7**. LC/MS^2 extracted ion chromatogram obtained from (A) BPA (300 μg L^{-1}) in acetonitrile
664 and (B) a contaminated tea soft drink with BPA (2.3 μg L^{-1}).
665
666

Figure 1

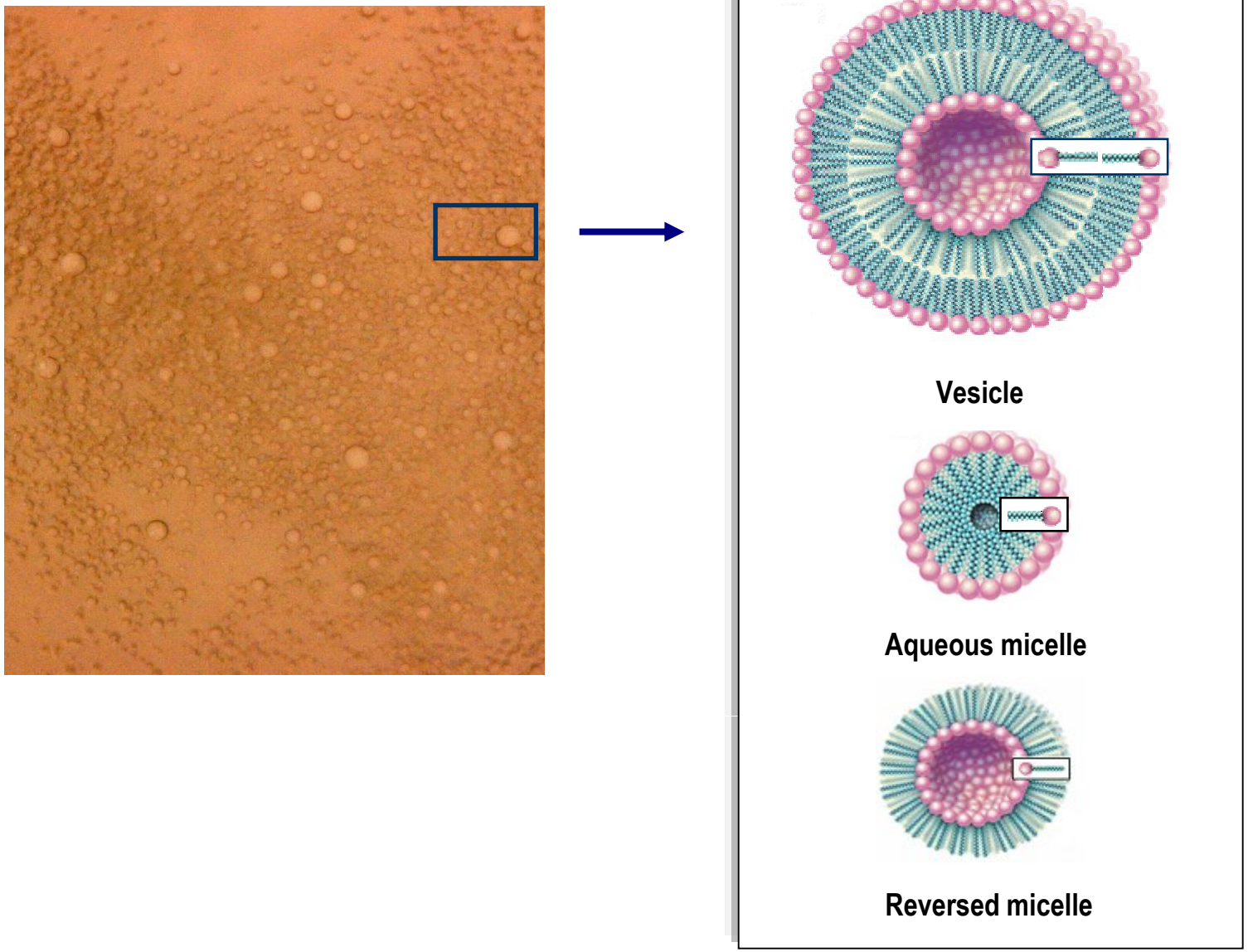


Figure 2

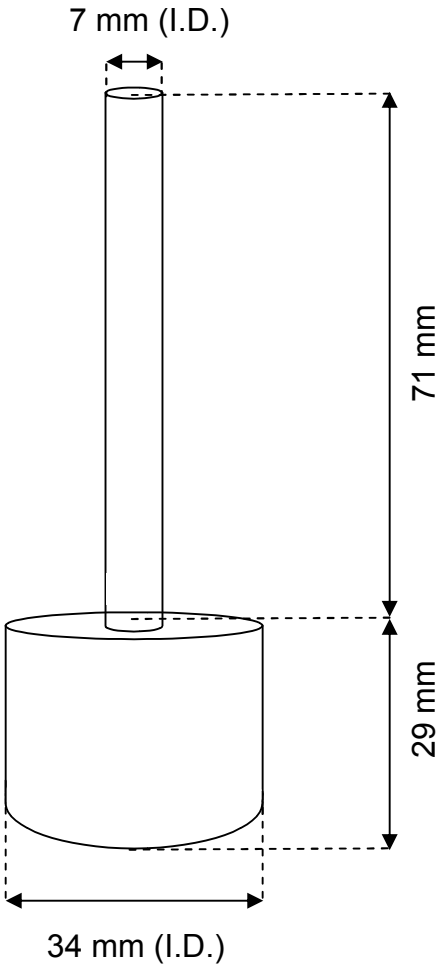
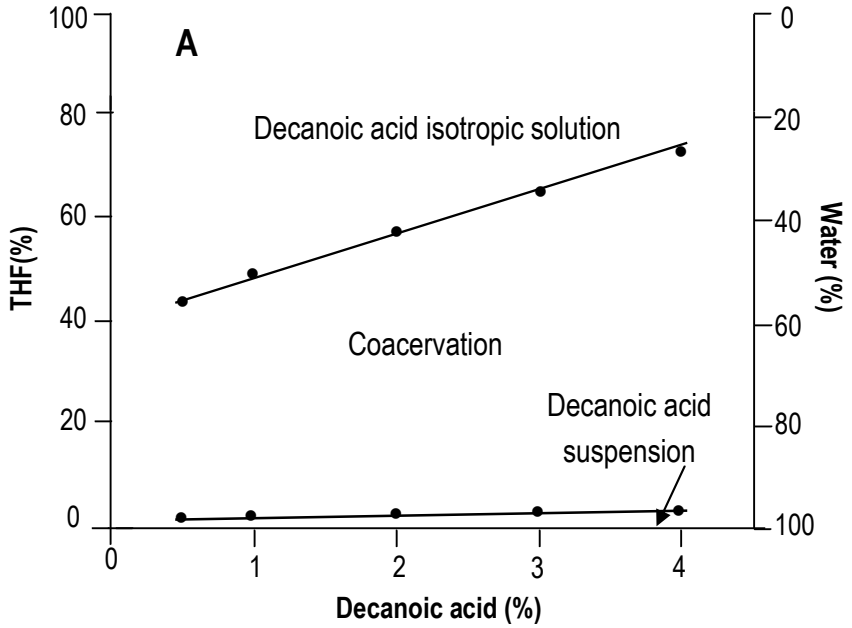


Figure 3


A
Decanoic acid isotropic solution
Coacervation
Decanoic acid
suspension
THF(%)
Water (%)
Decanoic acid (%)
100
80
60
40
20
0
0
20
40
60
80
100
0
1
2
3
4

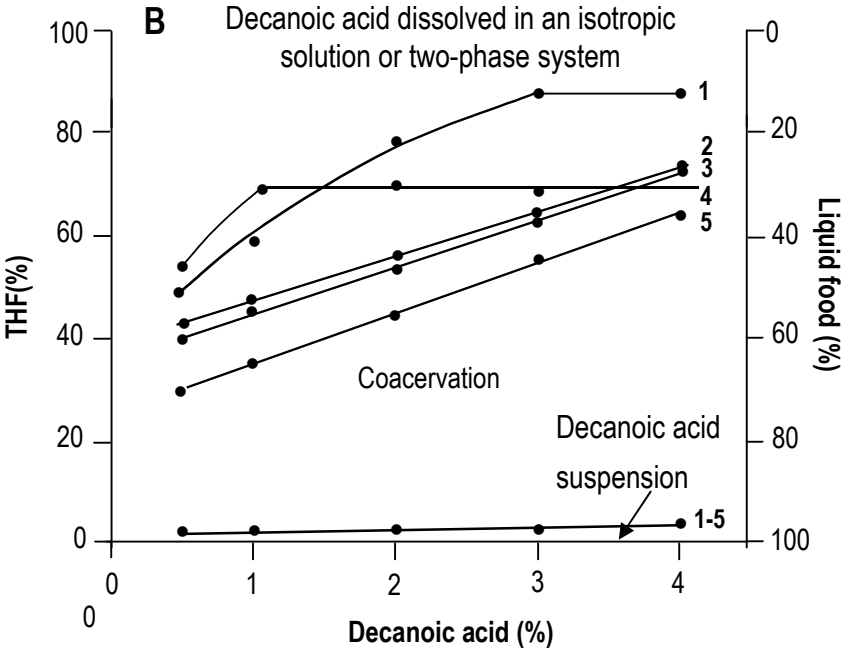
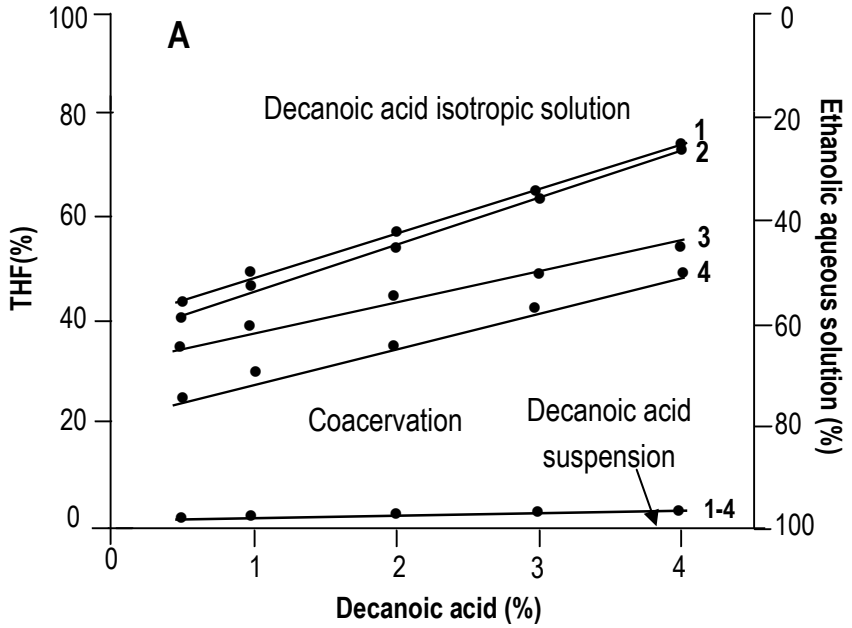

B
Decanoic acid dissolved in an isotropic
solution or two-phase system
Coacervation
Decanoic acid
suspension
1
2
3
4
5
1-5
THF(%)
Liquid food (%)
Decanoic acid (%)
100
80
60
40
20
0
0
20
40
60
80
100
0
1
2
3
4

Figure 4


A
Decanoic acid isotropic solution
1
2
3
4
Coacervation
Decanoic acid
suspension
1-4
THF(%)
Ethanolic aqueous solution (%)
Decanoic acid (%)
100
80
60
40
20
0
0
1
2
3
4

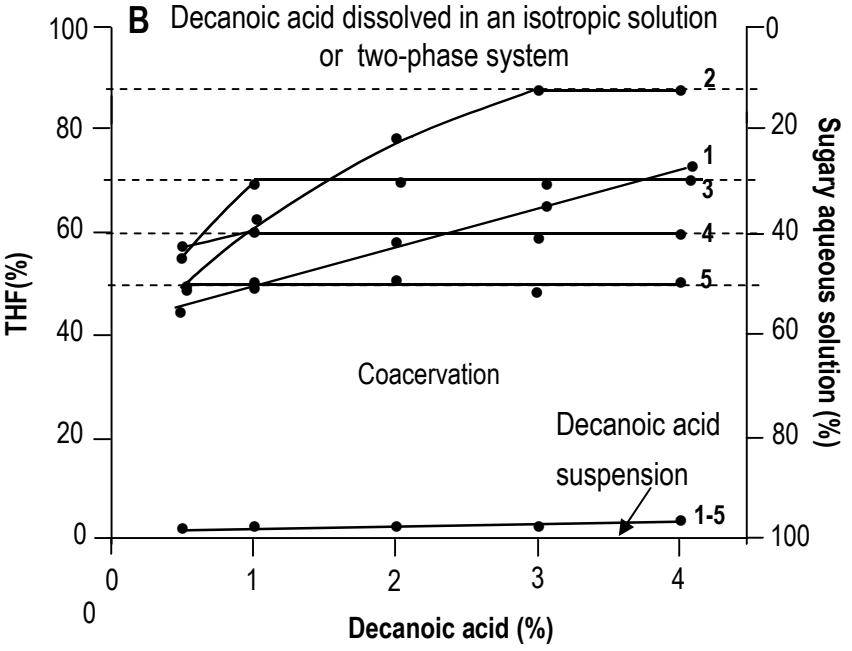

B
Decanoic acid dissolved in an isotropic solution
or two-phase system
2
1
3
4
5
Coacervation
Decanoic acid
suspension
1-5
THF(%)
Sugary aqueous solution (%)
Decanoic acid (%)
100
80
60
40
20
0
0
1
2
3
4

Figure 5

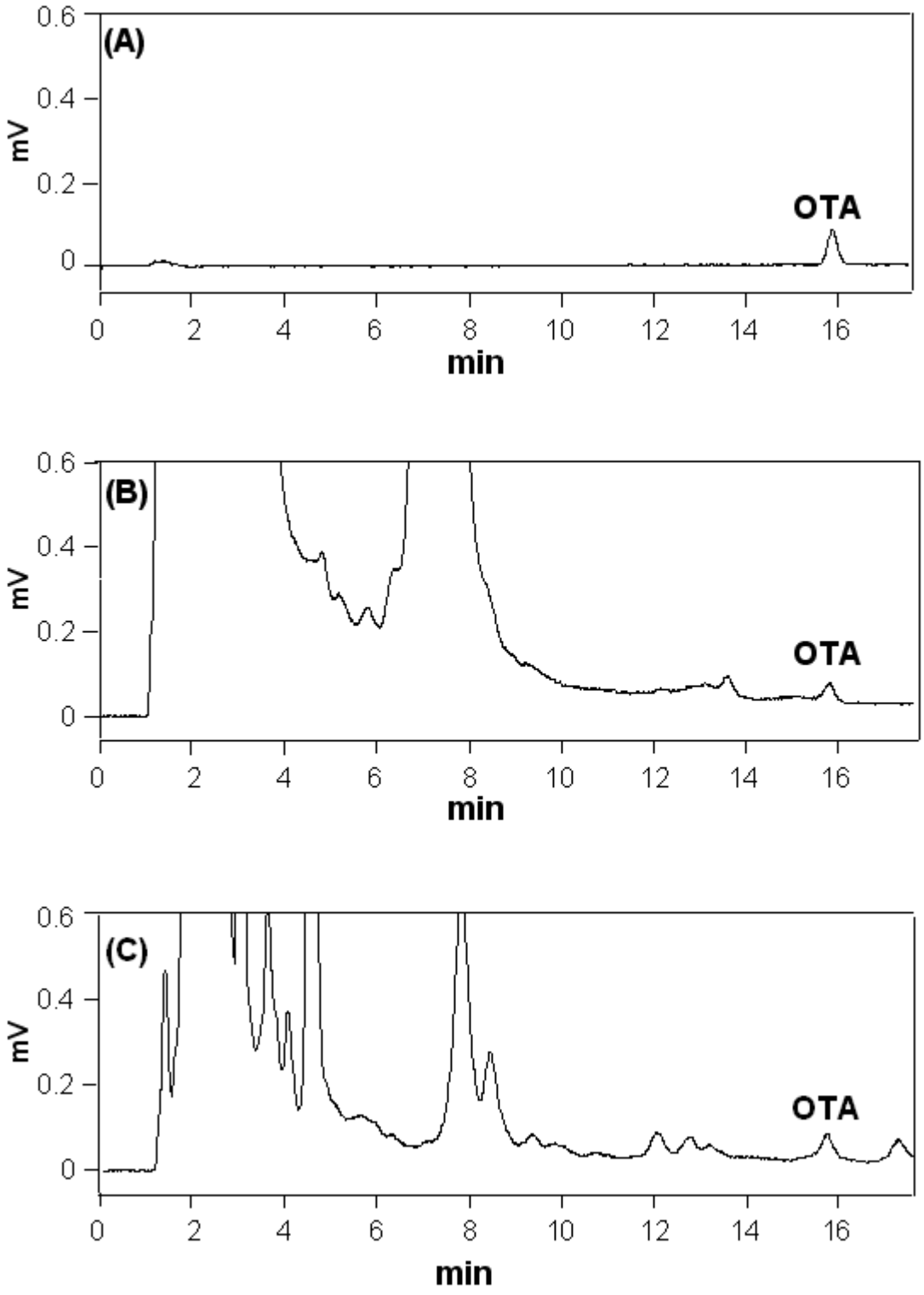


Figure 6

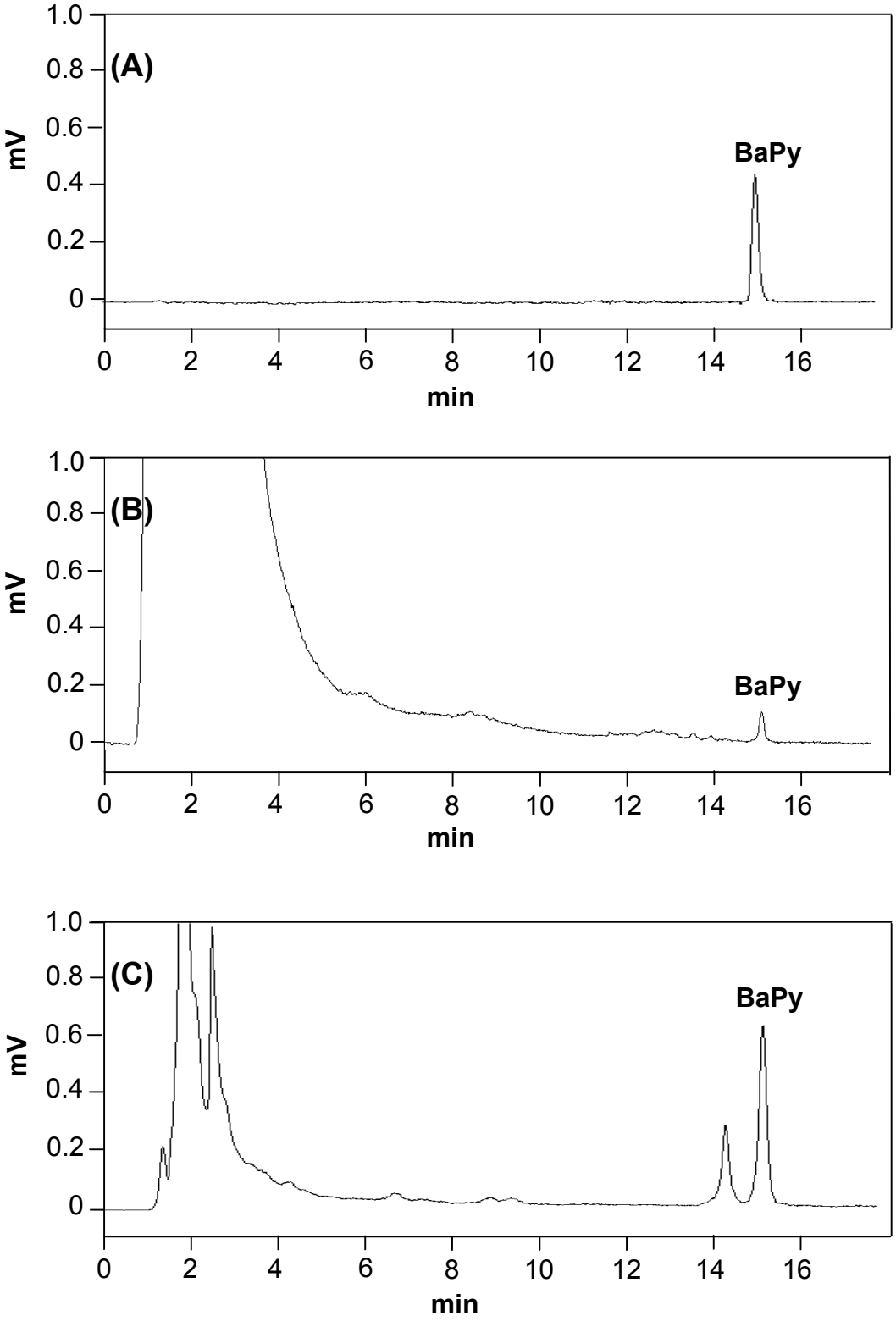

(A)
BaPy
mV
1.0
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
14
16
min
(B)
BaPy
mV
1.0
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
14
16
min
(C)
BaPy
mV
1.0
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
14
16
min

Figure 7

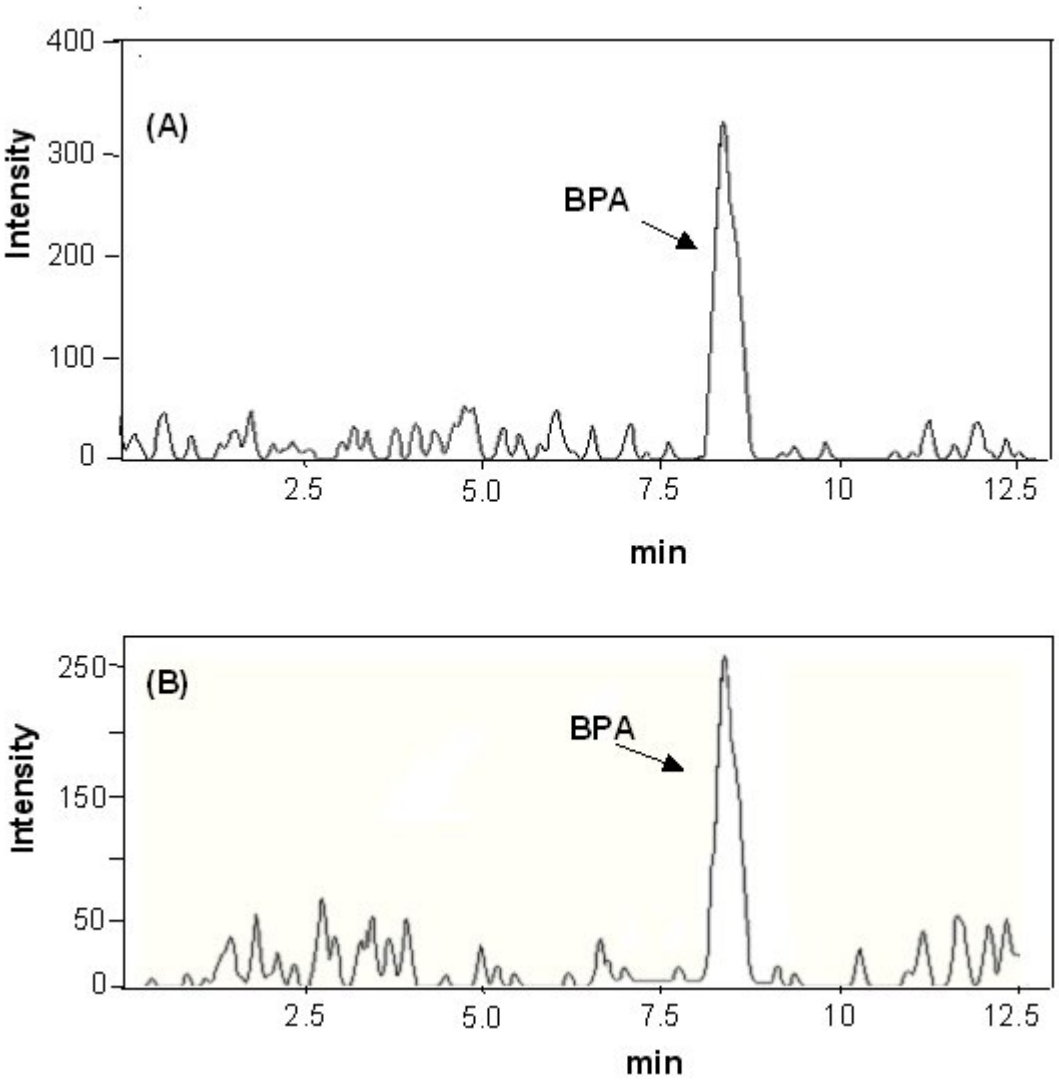
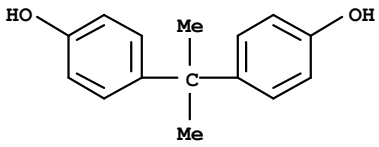
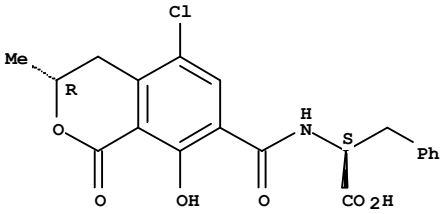
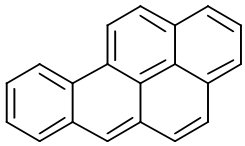

(A)
BPA
Intensity
400
300
200
100
0
2.5
5.0
7.5
10
12.5
min
(B)
BPA
Intensity
250
150
50
0
2.5
5.0
7.5
10
12.5
min

Table 1

Table 1

Structure, constants and number of hydrogen bonds for the target compounds

	Structure	[b]Log Kow	pK_a	[a]H Donor and Acceptor sum
Bisphenol A (BPA)		3.25	9.73	4
Ochratoxin A (OTA)		4.58	4.4 (acid group) 7.1(alcohol group)	10
Benzo(a)pyrene BaPy		6.40	-	0

[a]Calculated using Advanced Chemistry Development (ACD/Labs) Software V9.04 for Solaris

[b] Logarithm of the octanol-water partition coefficient

Table 2

Table 2

Composition of the liquid foods studied

Liquid food	**[a]Water (g mL^{-1})**	**[a]Protein (mg mL^{-1})**	**[a]Sugar (mg mL^{-1})**	**[b]Condensed tannins (mg mL^{-1})**	**[a]Ethanol (% v/v)**
Beer	0.97	3.60-4.48	0	0.022	3-5
Red wine	0.87	0.74	6.42	0.6471	10-13
White wine	0.87	0.74	9.93	0.0085	10-13
Vinegar of white wine.	0.90	0	0.38	-[c]	0
Must of white wine	0.93	0.62	164.49	0.0040	0
Red tea infusion	0.99	0	3.18	0.1423	0
Mate tea Infusion	0.99	0.03	0.03	0.1423	0
Soluble coffee brew	0.99	1	3.41	0.001	0
White soda soft drink.	0.97	0.54	97.68	-[c]	0
Lemon soft drink.	0.97	0.74	110.05	-[c]	0
Tea soft drink.	0.98	0	97.89	-[c]	0

Sources: [a] United States Department of Agriculture (USDA) National Database for Standard Reference, [b] USDA Database for the proanthocyanidin content of selected Foods.

[C] Foods not included in database, their condensed tannins content assumed to be undetectable.

Table 3

Table 3.

Figures of merits of the linear relationship (y = a+ bx) between the coacervate volume (y, μL) and the amount of decanoic acid (x, mg) for 10% of THF and the exponential relationships ($y = b_0 e^{b_1 z}$) between the coacervate volume (y, μL) and the concentration of THF (z, %) for 200 mg of decanoic acid.

Liquid food	**y = a+ b*x***			**$y = b_0 e^{b_1 z}$**		
	b ± SD(μL mg^{-1})	a ± SD(μL)	[a] R^2	b_0 ± SD (μL)	b_1± SD	[a]R^2
Water	1.67±0.04	0.7±3.9	0.997	205±7	0.045±0.002	0.996
Beer	1.79±0.07	5±26	0.994	220±8	0.046±0.005	0.980
Beer diluted 1:1 with water	1.68±0.04	3±10	0.991	207±6	0.045±0.005	0.990
Red wine	1.86±0.07	38±38	0.997	237±8	0.045±0.002	0.980
White wine	1.86±0.05	32±26	0.998	240±7	0.045±0.002	0.990
Vinegar of white wine.	1.64±0.10	7±7	0.990	201±5	0.044±0.006	0.991
Must of white wine	1.60±0.02	13±9	0.998	205±9	0.048±0.004	0.980
Red tea infusion	1.66±0.08	6±11	0.991	204±6	0.045±0.004	0.990
Soluble coffee brew	1.71±0.09	-8±10	0.993	210±10	0.046±0.007	0.991
White soda soft drink.	1.72±0.08	10±25	0.990	211±15	0.047±0.005	0.980
Lemon soft drink.	1.6±0.1	-2±20	0.980	200±4	0.046±0.005	0.992
Tea soft drink.	1.6±0.1	4±11	0.980	200±7	0.045±0.002	0.990

[a] correlation coefficient ; n =8

Table 4

Table 4

Mean percent recoveries and standard deviations (R ± SD, %) and actual concentration factors (ACF) obtained for OTA, BPA and BaPy in liquid foods as a function of decanoic acid concentration (%, w/v)

Contaminant	Food	Decanoic acid (%)											
		0.1		0.25		0.5		1		2		3	
		R±[a]SD	ACF	R±[a]SD	ACF	R±[a]SD	ACF	R±[a]SD	ACF	R±[a]SD	ACF	R±[a]SD	ACF
OTA	Water	60±3	307	83±4	175	93±4	100	98±1	50	98±2	26	100±3	18
	Wine	40±4	193	76±3	144	89±3	85	93±2	45	99±5	24	98±2	15
	Vinegar	44±3	241	84±3	184	93±5	102	98±4	54	101±4	27	99±3	18
	Must	55±4	309	84±4	189	92±4	104	99±2	56	98±5	27	99±3	18
	Beer	29±3	145	60±5	120	80±3	81	89±3	45	95±3	24	99±4	17
	Beer diluted 1:1 with water	39±4	99	70±4	97	89±4	48	93±2	26	98±4	13	99±2	9
BPA	Water	50±2	258	73±4	157	82±2	89	92±2	50	95±4	26	100±4	18
	White soda	27±2	140	64±3	133	79±3	83	87±3	45	92±2	24	97±3	17
BaPy	Water	64±3	330	90±3	192	95±4	103	100±2	54	99±2	26	101±2	18
	Tea infusion	30±3	162	82±5	178	93±4	93	95±3	51	98±3	26	98±3	18
	Soluble coffee	34±3	178	84±4	178	92±5	92	96±3	51	99±4	26	100±5	18

[a] *n*= 3; THF =10 %

Table 5

Table 5

Mean percent recoveries and standard deviations (R ± SD, %) and actual concentration factors (ACF) obtained for OTA, BPA and BaPy in liquid foods as a function of tetrahydrofuran concentration (%, v/v)

Contaminant	**Food**	**Tetrahydrofuran (%)**					
		5		**1 0**		**15**	
		R±[a]SD	ACF	R±[a]SD	ACF	R±[a]SD	ACF
OTA	Water	95±3	141	94±2	104	95±2	81
	Wine	89±3	112	90±2	86	80±2	58
	Vinegar	93±3	141	90±2	102	93±2	81
	Must	91±4	132	92±4	104	90±2	73
	Beer	70±5	96	81±3	83	83±3	65
	Beer diluted with water 1:1	89±5	65	89±3	49	91±3	39
BPA	Water	73±3	108	82±2	89	82±3	70
	White soda	70±3	98	79±3	83	80±3	62
BaPy	Water	91±3	135	95±4	103	100±2	85
	Tea infusion	90±2	133	92±4	93	99±2	84
	Soluble coffee	90±3	129	93±5	92	99±3	81

[a] *n*= 3; decanoic acid= 0.5%

Table 6

Table 6

Analytical performance of the methods developed for the analysis on BPA, OTA and BaPy in liquid foods

Target compound	Retention time (min)	Calibration			Method [b]LOQ (ng L^{-1})	Method [c]LOD (ng L^{-1})
		Linear range (μg L^{-1})	Slope±SD (L μg^{-1})	[a]r		
BPA	8.1	50-1000	80.02±0.09	0.995	562-602	200-215
OTA	15.8	2-5000	102.6±0.2	0.9998	14-18 (31 for beer)	4-5 (9 for beer)
BaPy	15.6	0.05-500	3460±5	0.998	0.37-0.39	0.11-0.13

[a]correlation coefficien; n=7; [b] estimated quantification limits of the method; [c] estimated detection limits of the method.

Table 7

Table 7

Mean concentrations (C± [a]SD, ng L^{-1}) and recoveries (R± [a]SD,%) along with their respective standard deviations found for OTA, BaPy and BPA in the analysis of liquid foods

Liquid food	OTA		BaPy		BPA	
	C ± SD	R ± SD	C ± SD	R ± SD	C ± SD	R ± SD
White wine, brand 1	n.d.	92±3 [b]	-	-	-	-
White wine, brand 2	n.d.	90±3 [c]	-	-	-	-
Red wine, brand 1	n.d.	90±3[b]	-	-	-	-
Red wine, brand 2	n.d.	92±7[c]	-	-	-	-
Vinegar (white wine), brand 1	< LOQ	90±5[b]	-	-	-	-
Vinegar (white wine), brand 2	92±5	93±4[d]	-	-	-	-
Must (white wine), brand 1	n.d.	91±3[f]	-	-	-	-
Must (white wine), brand 2	177±1	92±4[e]	-	-	-	-
Beer, brand 1	n.d.	81±2[f]	-	-	-	-
Beer, brand 2	115±4	79±2[e]	-	-	-	-
Mate tea infusion	-	-	16.6±0.7	94±4[g]	-	-
Red tea infusion, brand 1	-	-	4.9±0.2	94±4[h]	-	-
Red tea infusion, brand 2	-	-	n.d.	96±4[h]	-	-
Soluble coffee, brand 1	-	-	n.d.	91±5[i]	-	-
Soluble coffee, brand 2	-	-	1.51±0.01	90±5[j]	-	-
White soda soft drink (canned)	-	-	-	-	n.d.	82±6[k]
Lemon carbonated soft drink (canned)	-	-	-	-	<LOQ	80±3[k]
Tea beverage (canned), brand 1	-	-	-	-	n.d.	80±3[l]
Tea beverage (canned), brand 2.	-	-	-	-	2300±100	78±4[l]

[a] *n*=3; n.d.: non detected; <LOQ: below the quantification limit; Fortification levels: [b](25 ng L^{-1}); [c](65 ng L^{-1}); [d] (92 ng L^{-1}); [e] (150 ng L^{-1}); [f] (40 ng L^{-1}); [g](15 ng L^{-1});[h] (5 ng L^{-1}); [i] (0.7 ngL^{-1}); [j](1 ng L^{-1}); [k](1000 ng L^{-1}); [l] (5000 ng L^{-1})