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Antimony Speciation in Spirits Stored in PET Bottles: Identification of a Novel Antimony Complex†

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Total antimony and its +V and +III oxidation state species were determined in twelve spirit samples (Greek *raki* and *tsipouro*) stored in polyethylene terephthalate bottles. Reliable and reproducible results were obtained following direct analysis by using ICP-MS providing total Sb concentrations between 0.4 – 4 μg L^{-1}. Antimony speciation analysis by LC-ICP-MS was also assessed, showing the presence of both inorganic Sb species along with an unknown Sb complex, which was the predominant species in all samples analysed. The structure of this complex was investigated by using liquid chromatography with high-resolution tandem mass spectrometry. The analysis gave evidence for an acetaldehyde-bisulphite pyruvate Sb complex with the formula: $C_7H_{14}O_{12}S_2Sb$. The proposed ligands are organic substances expected to be present in the *raki* matrix. In addition, the influence of high temperature storage conditions and extended exposure times up to two weeks, on Sb migration from PET bottles into *raki* samples was investigated. Total Sb and Sb species content was determined by ICP-MS and LC-ICP-MS, respectively. The concentrations determined were in the range of 5.6 to 28 μg Sb L^{-1} spirit after a week of storage at 60 ºC. In which case, inorganic Sb(V) and Sb(III) became the predominant species in comparison to the "novel" organic Sb complex.

Introduction

Polyethylene terephthalate (PET) is the fastest growing plastic used for food packaging applications as a result of its popular use for replacing glass containers. Specifically, PET is used in all sizes of soft drinks and mineral water bottles, which are produced by injection stretch blow moulding.[1–3] PET is also thermoformed to make trays and pots used for cooking and heating foods in both conventional and microwave ovens.

Food packaging has the main function of protecting food from contamination. This necessitates the requirement that the packaging material is inert enough to not cause food contamination during the entirety of its contact period, from the production process until final consumption of the packaged food. For this reason migration mechanisms involving chemicals present in the packaging to the food matrices need to be studied in detail. In the case of PET, significant amounts of antimony (Sb) can be found in the plastic, which come from the catalyst in the form of antimony trioxide or triacetate used during the polymer manufacturing.[3,4] Therefore, due to the common use of PET as a packaging material by the food industry, Sb frequently comes into contact with food and drink. Consequently, Sb has been observed at slightly elevated concentrations in products for human consumption.

The toxicological properties of Sb depend on its chemical form and oxidation state. The semimetal is found in organic and inorganic compounds in two oxidation states. For the inorganic forms, Sb(III) is 10 times more toxic than Sb(V).[5,6] The Sb complexes with organic ligands are considered to be less toxic than inorganic Sb. In addition, Sb is considered a priority pollutant by the European Union (EU)[7] and the United States Environmental Protection Agency (USEPA).[8]

Several studies have reported on the determination of total Sb concentration in PET packaging, most of which are used for mineral water storage. Values between 100 and 400 mg kg^{-1} have been reported.[9–19] This is due to significant remains of the semimetal on the plastic polymer chain once the manufacture of PET is finished.[3,11,14]

In addition to the extensive use of PET for water storage, in some countries there is an increasing trend for reuse of PET containers for the storage of various foodstuffs, especially bulk goods. A common practice is the storage of alcoholic beverages in PET bottles. Although alcoholic beverages tend to be stored in glass containers, in some countries, such as Greece, it is typical to store local homemade spirits, such as *raki* or *tsipouro*, in PET bottles in order to ease their transport.

Greek *raki* (ρακή) or *tsikoudia* is an alcoholic beverage made on the island of Crete by distilling pomace (pieces of grapes including stems and seeds) pressed during the winemaking process. The resulting spirit is similar to tsipouro made on the mainland of Greece. These spirits do not contain any aniseed as does the Greek drink ouzo or the Turkish raki.[20] These are similar to other Mediterranean alcoholic beverages, such as Spanish pomace and Italian *grappa*, in terms of organoleptic properties, chemical composition and manufacturing

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techniques. The final ready-to-consume product of *raki* or *tsipouro* contains approximately a 40-65% of alcohol by volume.[21]

Spirits present a complex matrix due to the presence of different organic acids, among other organic substances. This fact, among others, may promote the migration of Sb from the PET bottle to the beverage, leading to concentrations in the order of μg L^{-1}. In this way, Sb migration studies from PET into food products is a good tool for providing useful information regarding the potential risk associated with the presence of antimony under different storage conditions. It has been reported that several physicochemical factors influence antimony migration, among which storage time and temperature predominate, in addition to factors like the type of PET and matrix characteristics of the beverage.[22] Following migration, Sb is found in the beverage or spirit as inorganic Sb(V) and Sb(III), as well as in complexed forms, depending on the matrix composition.

Since limited information has been published on the determination of metal species in alcoholic beverages, in particular there is no information about the determination of antimony (total and species) in PET bottled spirits, this paper deals with the study of the presence of antimony in PET bottled Greek *raki* and *tsipouro*. As this type of sample has a complex matrix and a significant alcohol content, the analysis of total Sb and species concentration was assessed, together with the elucidation of the structure of the Sb species present. In addition, since scarce information regarding the Sb migration processes in PET bottled beverages has been found, the Sb migration behaviour at elevated storage temperature in *raki* samples has also been investigated in the present paper.

Experimental

Instrumentation

Total Sb content was determined using inductively coupled plasma mass spectrometry (ICP-MS) *Perkin Elmer NexION 300XX* model with Rh as the internal standard. The ion intensity at m/z 121 (^{121}Sb) was monitored. A quaternary pump HPLC system (*Perkin Elmer Flexar*) equipped with an autosampler with an injection volume between 0.01 and 2000 μL and an anion exchange column was coupled to the *NexION 300X* ICP-MS for Sb speciation. For the instrument control and data acquisition *Chromera* software was used.

The experimental conditions used with the LC-ICP-MS were the following: PRP-X100 column (125×4.1 mm Hamilton, 10 μm particle size, USA) operated at room temperature using mobile phases of 10 mM ethylenediaminetetraacetic acid (EDTA) at pH 4.0 with 0.5% methanol at a flow rate of 1.5 mL min^{-1} (LC Method A) or 10 mmol L^{-1} ammonium formate with 2 % of methanol at a flow rate of 1.0 mL min^{-1} (LC Method B).

Molecular mass spectrometry was used for the characterization of the structure of antimony complexes. A *Thermo Scientific LCQ Advantage* ion trap mass spectrometer with a sonic spray ionisation (SSI) source was used. The SSI source has been reported to be an energetically mild ionization source for metal complexes, suitable for analysing easily oxidized metal species, as well as capable for providing simultaneous negative and positive ion generation. The applied SSI conditions are detailed elsewhere.[23]

For antimony species characterisation, a liquid chromatography system was coupled to the SSI-MS by a PEEK sleeve. A quaternary pump model *Shimadzu LC-20AD* equipped with a manual stainless steel sampler injector (Rheodyne Model 7125i) with a 2 μL loop. The separation of antimony species was performed in analytical RP-C18 columns Shim-pack XR-ODS. The mobile phase used was a mixture of methanol and 0.1% formic acid at flow 0.1 mL min^{-1}. For the instrument control and data acquisition *Xcalibur* software was used. Whereas for structural elucidation using High Resolution Mass Spectrometry (HRMS), a *Thermo Fisher Scientific LTQ Orbitrap Velos* equipped with a thermally assisted electrospray ionization source was used. The instrument was operated in negative mode, applying the following parameters: ESI voltage was -2.5 kV; capillary and vaporizer temperatures were 320ºC; sweep gas, auxiliary gas and sheath gas flow rate were 2, 10 and 40 arbitrary units, respectively, and the tube lens was held at 50 V. For tandem MS experiments, the normalised collision energy (NCE) ranged from 20 to 50%, depending on the ion, and nitrogen was used as collision gas (1.5 mTorr).

Reagents and standards

Ultrapure water with 18 M Ω cm^{-1} conductivity (Millipore, Bedford, MA, USA) was used for making up volume standards and reagents. 1000 mg L^{-1} stock standard solutions of Sb(III) and Sb(V) were prepared by dissolving appropriate amounts of potassium antimony (III) oxide tartrate hemihydrate (Fluka, Neu-Ulm, Germany) and potassium hexahydroxoantimonate (Riedel de-Haën, Seelze, Germany) in water, respectively. The stock standard solutions were stored in polyethylene bottles at 4°C. The working standard solutions were prepared daily by dilution in the mobile phase used for speciation analysis and in acidic medium (nitric acid, Panreac) for total analysis.

The mobile phases were prepared daily by dissolving EDTA, ammonium formate or formic acid (Panreac) in water. In the case of the EDTA mobile phase, the pH was adjusted with dilute ammonia (Panreac) and then passed through a 0.45 μm filter (Millipore type HA).

Selection of samples

A total of 12 spirit samples from local stores and suppliers were selected for the determination of Sb: 9 samples of Greek *raki* (*tsikoudia*) and 3 samples of Greek *tsipouro*. Among these, 2 samples of both *raki* and *tsipouro* were stored in glass bottles for comparison purposes in terms of antimony concentration. Total Sb and species content were determined by ICP-MS and LC-ICP-MS, respectively. Among these samples, 2 PET bottled *raki* samples were selected for the determination of Sb by mass spectrometry and 6 PET bottled *raki* samples were further selected for the performance of migration studies under controlled temperature conditions.

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Determination of antimony

Total antimony in *raki* by ICP-MS

Samples were pumped into the nebulizer together with the internal standard (2% HNO_3 acid) by means of a T-piece mixer. An additional line was used for waste removal. The samples were quantified by means of an external calibration curve using total Sb standards from 0.1 μg L^{-1} to 10 μg L^{-1}.

Antimony speciation by LC-ICP-MS

Samples were directly injected to the LC-ICP-MS system under the conditions aforementioned. Antimony species in the extracts were identified by comparison of retention times with those of standards. Antimony in the samples was quantified by means of external calibration curves using Sb(III) and Sb(V) standards from 0.1 μg L^{-1} to 10 μg L^{-1}.

Mass spectrometric analysis of unknown antimony species

For the operation of SSI-MS, a 15 – 20 cm long polyimide coated fused-silica capillary (100 μm id. × 200 μm od) was inserted into the pneumatic nebulizer's sample uptake channel till it is aligned with the glass nebulizer spray tip. The fused silica capillary is secured in place by tightening a PEEK sleeve around it in the back of the nebulizer. The tip of nebuliser was placed approximately 1 – 3 mm from the mass spectrometer orifice. The temperature of the heated ion transfer capillary was set to 300ºC, the source induced dissociation (SID) voltage applied was 5V, the collision energy was 20% and the nitrogen gas pressure used for nebulization was between 40-60 psi.[23] For HRMS analysis, samples were analysed by direct infusion using a syringe and a syringe pump at a flow rate of 10 μL min^{-1}.

Computational Details of unknown antimony species

Several molecular models of the Sb complexes were created in order to explore the variety of the possible structures of the same molecular mass but with different connectivity of the atoms by conducting quantum chemistry calculations. Sets of possible ligands obeying the corresponding chemical composition $C_7H_{14}O_{12}S_2Sb$ (as determined using high resolution mass spectrometry) were created based on chemical intuition in order to build these models. Moreover, both the Sb oxidation states III and V were taken into account during calculations. The prediction of the geometries of the Sb complexes was based on the Density Functional Theory (DFT) method and the RI-B-P/def2-SVP computational model was used during calculations.[24] Tight convergence criteria were used for both the self-consistent field procedure (10^{-7} au) and the gradient (10^{-4} au). The corresponding auxiliary basis sets were used for the Resolution of Identity (RI) approximation.[25] During the optimization of the geometry, no symmetry restrictions were applied. The calculations were performed with the Turbomole quantum chemistry package.[26]

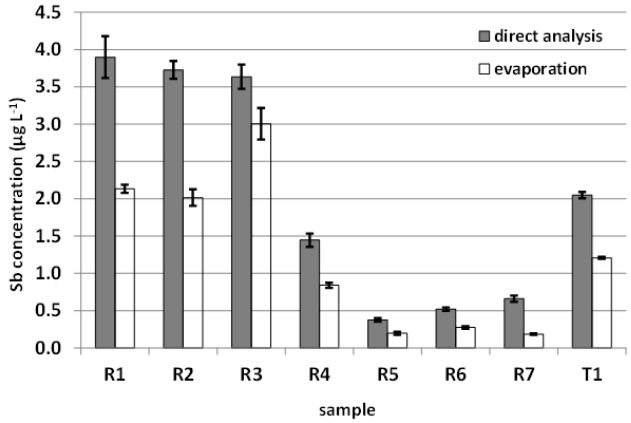

Figure 1 Total Sb concentration in PET bottled *raki* and tsipouro samples. R1 – R7: PET bottled *raki*; T1: PET bottled tsipouro (n=3).

Results and Discussion

Analysis of total Sb by ICP-MS

Total Sb content was determined in all samples using 2 different sample preparation approaches: direct analysis following sample dilution and analysis after sample evaporation and appropriate dilution. Both approaches we performed in order to reduce the ethanol content in the samples and thus prevent plasma instability. The evaporation process was performed in a water bath at 50 ºC for 1 hour. Prior to analysis, samples and evaporated samples were diluted four and two times, respectively, in acidic media.

The Sb content in glass bottled samples was found to be lower than the limit of detection (LOD was in the low ng Sb per L range), whereas PET bottled samples showed significantly higher concentrations. The Sb concentrations determined in the PET bottled samples are plotted in Figure 1. Antimony concentration differences were observed for the same samples that had been prepared using the two different approaches: the concentrations obtained with the direct analysis approach were between 0.4 – 4 μg L^{-1}, whereas the values obtained using the evaporation process were systematically lower for all samples: 0.2 – 3 μg L^{-1}. RSD values were ≤ 10 in all cases. The direct analysis of samples following dilution gave slightly higher Sb concentrations, which could be due to a matrix enhancement effect caused by the ethanol or the possible loss of the Sb content during the alcohol evaporation process for the evaporated samples. It was observed that more than half of the PET samples showed concentrations higher than 1 μg L^{-1}. These values are higher than those typically obtained for water samples stored in PET containers, which have been reported in previous work.[19] This difference could be partially related to the samples' high ethanol content as well as the presence of potential organic ligands that may be promoting Sb extraction. Ethanol is used as a food simulant for alcoholic foodstuffs to perform the migration test stipulated in the EU Directive 97/48/CE.[27] Spirits typically have a high content of fusel alcohols, organic volatile molecules and sugar, which could ease or promote the migration of Sb from the PET matrix. Moreover, 3 PET bottled *raki (tsikoudia)* samples had Sb concentrations close to 4 μg L^{-1}, which is quite close to the limit established by the EU for drinking waters (5 μg L^{-1}). However, as consumption of such spirits is relatively low for the general public their Sb

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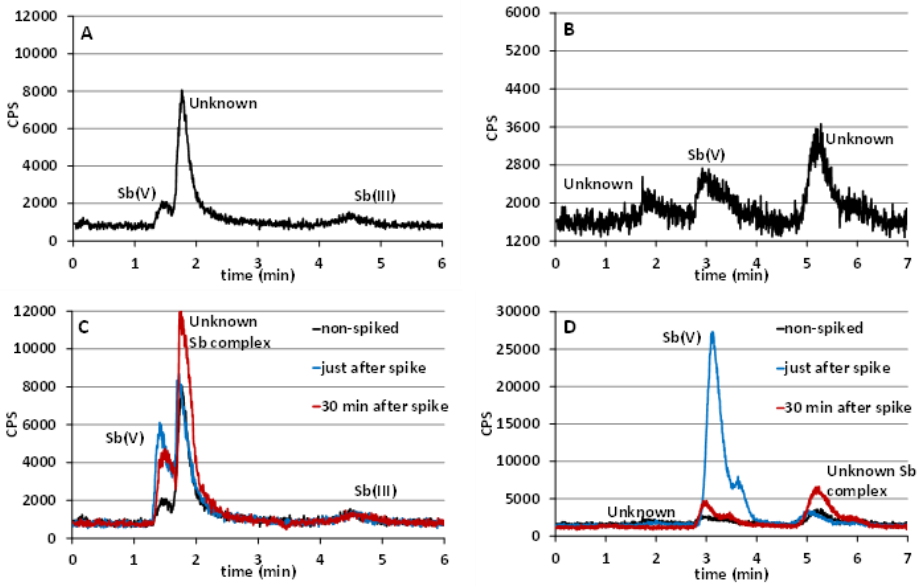

Figure 2 Chromatograms obtained from *raki* R2 by LC-ICP-MS using EDTA 10 mmol L^{-1} pH 4 with 0.5% of methanol and ammonium formate 8 mmol L^{-1} with 2% of methanol as mobile phase (A, B) and spike tests with 2 µg L^{-1} of Sb(V) (C, D).

content does not seem to be of significant concern for public health. In addition, in the present study these spirits have been stored in the PET bottles for over 12 months prior to analysis. It is our ambition, however, to try and further understand the physicochemical processes responsible for Sb migration from the PET material to the spirit.

Antimony speciation by using LC-ICP-MS

After the determination of total Sb in *raki* and *tsipouro*, Sb speciation in the PET bottled samples was carried out by using LC-ICP-MS. Two different separation conditions both based on anion exchange chromatography were assessed for the separation of Sb species. The first method used a mobile phase containing EDTA 10 mmol L^{-1} at pH 4.0 with 0.5 % of methanol run at a flow of 1.5 mL min^{-1} (*LC Method A*). This approach has already been described elsewhere.[19] The second separation method used a mobile phase containing ammonium formate 10 mmol L^{-1} with 2 % of methanol run at a flow of 1.0 mL min^{-1} (*LC Method B*). This mobile phase has previously been used for the separation of several Sb(V) complexes in Sb spiked yogurt samples.[28] Inorganic Sb(V) and Sb(III) standards were also analysed using both separation methods in order to help identify the Sb species detected in the *raki* samples.

The chromatograms resulting from monitoring Sb in the *raki* samples contained a maximum of 3 Sb peaks for each of the chromatographic methods. As an example, Figure 2 A and B depicts the chromatograms obtained from the analysis of sample R2 using LC Methods A and B, respectively. When using EDTA as the mobile phase (LC Method A), the first and the third peak have the same retention times as does inorganic Sb(V) and Sb(III) standards, respectively. Thus, *raki* peaks 1st and 3rd correspond to non-complexed Sb(V) and Sb(III) species, respectively. The inorganic Sb(III) species is only present in *raki* samples R1 – R3. The second peak, which is the main Sb species in all the analysed *raki* samples, has a retention time slightly longer than inorganic Sb(V), corresponding to an unknown Sb species. The Sb peaks obtained using LC Method B correspond to Sb(V) species, as inorganic Sb(III) does not elute using these separation conditions.[28] The second peak, observed using LC Method B, has the same retention time as the Sb(V) standard, therefore, it corresponds to non-complexed Sb(V) species (Fig. 2 B). The other two peaks may correspond to unknown complexes of Sb.

To further identity of the Sb species present in the *raki* samples, tests were performed by spiking with Sb(V) and Sb(III) standards. Samples were spiked with a concentration of 2 µg Sb L^{-1} using each Sb species. On the one hand, it was verified that the third peak observed when using LC Method A corresponds to Sb(III) species, as the intensity increased when spiking with inorganic Sb(III). On the other hand, samples spiked with Sb(V) were analysed using each of the two LC methods A and B. The results obtained are depicted in Figure 2 C and D, respectively. When samples were analysed using LC Method A just after spiking with Sb(V), only the intensity of the non-complexed Sb(V) increased, as can be seen with respect to the non-spiked sample (Fig. 2C). However, when repeating the analysis 30 minutes later, the signal of Sb(V) decreased whereas the intensity of the second peak (unknown Sb species) increased. When using LC Method B the third peak was observed to increase slightly.

Moreover, it should be taken into account that the loss and gain of the area of Sb(V) and the unknown Sb species, respectively, 30 min after spiking, is different for each of the two separation methods. When using the EDTA mobile phase (LC Method A), although the signal gain for the unknown species is slightly higher than the signal loss for Sb(V) species, the % gain and loss, respectively, in terms of area and concentration are similar. However, with the ammonium formate method (LC Method B), the signal loss for non-complexed Sb(V) is much more significant than the signal gain for the unknown species,

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which demonstrates that there is a loss of the unknown Sb species when using LC Method B. Thus suggesting that a significant portion of the unknown Sb species may be non-eluting Sb(III) species.

From the results obtained, the use of the EDTA mobile phase gave better elution performance. However, when using other detection systems such as electrospray ionization (ESI) -MS, the use of ammonium formate (LC Method B) is to be preferred in order to avoid problems related to the use of non-volatile salts or buffers.

Once the most suitable separation method was selected (LC method A) , i.e. giving the highest recovery, Sb species in the *raki* samples were quantified by means of external calibration curves of Sb(V) and Sb(III), which were prepared in 40% of ethanol to simulate the sample matrix. As the unknown species has a retention time very similar to the Sb(V), it was quantified using the Sb(V) calibration curve. Column recoveries calculated with respect to total Sb contents ranged from 70 – 110% except for sample R1, which gave a slightly lower value (62%). This fact could be due to the presence of other minor Sb species that did not elute from the column. RSD values obtained were ≤ 10% in all cases. However, for three samples (R5 – R7) with total Sb content around 0.5 µg L^{-1} or even lower, high recovery values were obtained, which demonstrates the analytical challenge resulting from the extended sample dilution needed for the analysis. Even though the unknown species, which is the predominant Sb species in all samples, was quantified using a different standard, the Sb species recoveries obtained for five of the samples analysed were acceptable.

Analysis of unknown Sb complex by using LC-SSI- ion trap low resolution MS and direct infusion ESI- orbitrap high resolution MS

Optimization of the separation process

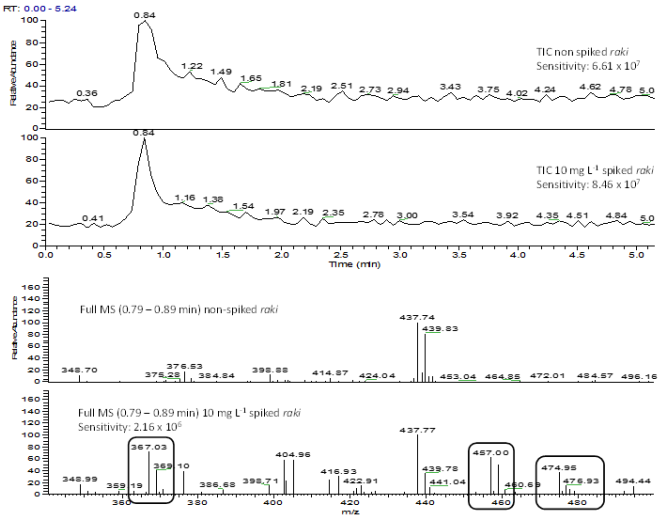

Figure 3 TIC of non-spiked and 10 mg Sb(V) L^{-1} spiked *raki* R1 together with the average spectra of the 0.84-minute peak signal.

The analysis of Sb species by LC-ICP-MS showed that *raki* samples had an unknown Sb complex as their major Sb species. In order to determine the structure of this complex, several studies by SSI-MS were performed by using ammonium formate as mobile phase.

First of all, the two *raki* samples with the highest concentration of Sb, R1 and R2, were selected and analysed by ESI-MS with direct infusion at the conditions described in section 2.4. The samples were analysed both directly and spiked at different concentration levels of Sb(V): 10 µg L^{-1}, 100 µg L^{-1}, 1 mg L^{-1} and 10 mg L^{-1}. The spectra showed plenty of ion signals and a high level background in all cases from which it was not possible to identify any Sb-containing ions. Therefore, it was decided to analyse the samples by LC-SSI-MS with the

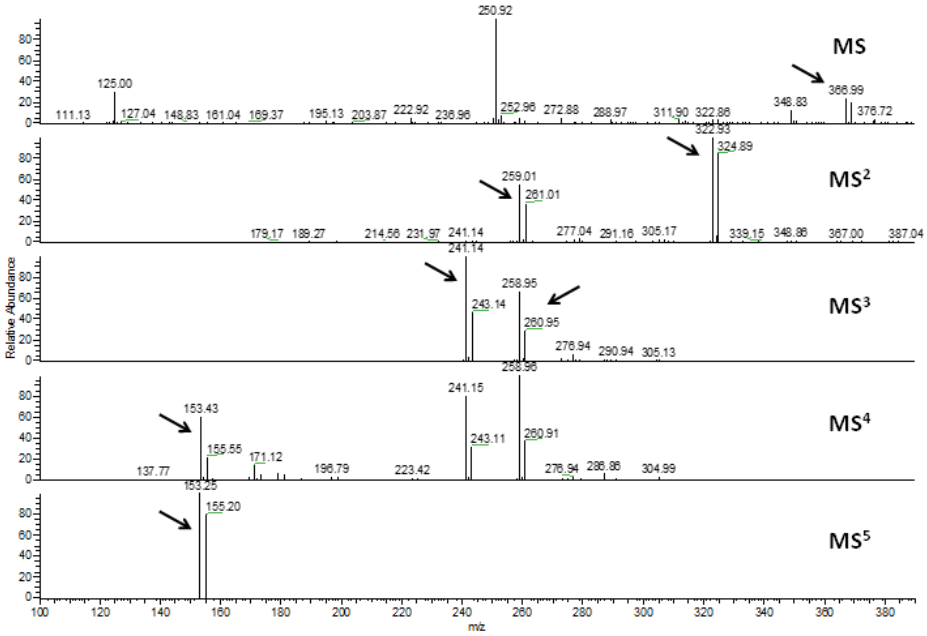

Figure 4. MS, MS^2 on m/z 367, MS^3 on m/z 259, MS^4 on m/z 241 and MS^5 on m/z 153 spectra of the organic Sb complex in *raki* R1 spiked sample with 10 mg L^{-1} of Sb(V). Collision energy for MS and MS^2: 20%. Collision energy for MS^3 and MS^4: 25%.

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Table 1. Assignment of prominent ions observed for the unknown Sb complex in raki spiked to contain 10 mg Sb L-1.

MS^n n	Molecular ion (m/z)	Elemental Composition / Proposed Formula	Product ion (m/z)	Product ion Assignment
2	474.89456-476.89502	$C_7H_{14}O_{12}S_2Sb$ $[Sb(C_2H_5O_4S)_2(C_3H_3O_3)(OH)]^-$	456.88419-458.88443	$[M - 18]^-$ $[M - H_2O]^-$
3	456.88419-458.88443	$C_7H_{12}O_{11}S_2Sb$ $[Sb(C_2H_5O_4S)(C_2H_3O_3S)(C_3H_3O_3)(OH)]^-$	366.90710-368.90755	$[M - 90]^-$ $[M – (C_2H_2O_2S)]^-$
			348.89706-350.89719	$[M - 108]^-$ $[M – (C_2H_4O_3S)]^-$
4	366.90710-368.90755	$C_5H_{10}O_9SSb$ $[Sb(C_2H_5O_4S)(C_3H_3O_3)(OH)_2]^-$	322.88165-324.88183	$[M - 44]^-$ $[M – (C_2H_4O)]^-$
			258.91977-260.92005	$[M - 108]^-$ $[M – (C_2H_4O_3S)]^-$
4	348.89706-350.89719	$C_5H_8O_8SSb$ $[Sb(C_2H_3O_3S)(C_3H_3O_3)(OH)_2]^-$	322.88165-324.88183	$[M - 26]^-$ $[M – (C_2H_2)]^-$
5	322.88165-324.88183	$C_3H_6O_8SSb$ $[Sb(HO_3S)(C_3H_3O_3)(OH)_2]^-$	304.87091-306.87125	$[M - 18]^-$ $[M - H_2O]^-$
			258.91977-260.92005	$[M - 64]^-$ $[M – (O_2S)]^-$
	304.87091-306.87125	$C_3H_4O_7SSb$ $[Sb(HO_3S)(C_3H_3O_3)(O)]^-$	240.90912-242.90948	$[M - 64]^-$ $[M – (O_2S)]^-$
	258.91977-260.92005	$C_3H_6O_6Sb$ $[Sb(C_3H_3O_3)(OH)_3]^-$	240.90912-242.90948	$[M - 18]^-$ $[M - H_2O]^-$
	240.90912-242.90948	$C_3H_4O_5Sb$ $[Sb(C_3H_3O_3)(OH)(O)]^-$	152.89354-154.89395	$[M - 88]^-$ $[M – (C_3H_4O_3)]^-$
6	152.89354-154.89395	O_2Sb $[Sb(O)_2]^-$	-	-

conditions described in sections 2.1 and 2.4. The mobile phase selected was a mixture of 0.1% formic acid and methanol 80:20 and the column length was 3 cm (reversed phase HPLC). The results showed a peak signal with the same retention time as the dead volume (0.8 min). Figure 3 shows the Total Ion Chromatogram (TIC) of the non-spiked *raki* R1 and the spiked one at 10 mg L^{-1} level together with the corresponding spectra average of the peak signal. For samples spiked with 1 mg Sb L^{-1}, this signal corresponded to antimony complexes with mass-to-charge ratio (m/z) of 367-369, 457-459, 475-477. In the non-spiked sample and the spiked ones at a concentration level lower than 1 mg L^{-1}, no ion signals matching the isotopic distribution of Sb were observed.

In order to improve the retention of the Sb complex on reversed phase chromatography two chromatographic parameters were assessed and modified: the composition of the mobile phase and the column length. First of all, *raki* sample R1 spiked with 10 mg L^{-1} of Sb(V) was analysed using different ratios of formic acid - methanol. The ratio of formic acid 0.1% solution was increased from the fixed value used in the previous test (80%) to 100%. As the percentage of methanol decreases, the retention of the Sb peak increases slightly, indicating some hydrophobic interaction with the reversed phase stationary phase. However, for organic solvent content lower than 10%, the Sb peak shows deformation. Thus, the optimum mobile phase ratio arrived at was 90% of 0.1% formic acid and 10% of methanol.

To further increase retention and mass spectrometric sensitivity a 10-cm length column was assessed and the results obtained were compared to those obtained using the 3-cm length column. The spiked R1 sample was analysed and the mobile phase with the optimum ratio (90% of formic acid and 10% of methanol) was used. As expected, retention time for the unknown Sb peak increased significantly. These were the optimum conditions used for the analysis of the unknown Sb species by LC-SSI-MS.

Characterisation of unknown Sb complex

After the assessment of the optimum conditions for the determination of the unknown Sb complex in *raki* spiked with Sb(V), the confirmation and identification of the corresponding Sb species was carried out by studying the fragmentation of the complex by using the tandem mass spectrometry. First of all, the ion with m/z 367 was selectively monitored and its product ion mass spectra recorded. The obtained fragments containing ions with the Sb isotopic distribution were further selected and fragmented in subsequent tandem mass spectrometric experiments. Tandem mass spectrometric experiment MS^n (n=2-5) were therefore performed. Figure 4 summarises the mass spectra obtained by tandem mass spectrometry.

During MS^2, it can be observed that the complex with m/z 367-369 yielded the ions at m/z 323-325 and 259-261 with the isotopic distribution typical of the Sb (even though some mass bias in the ion trap did not give the exact isotope abundances). In MS^3, the product ion with m/z 323-325 yielded the ion at m/z 259-261 and the product ion with m/z 259-261 yielded the ion at m/z 241-243. In MS^4, the product ion with m/z 241-243 yielded the ion at m/z 153-155. Sb atomic ions were not

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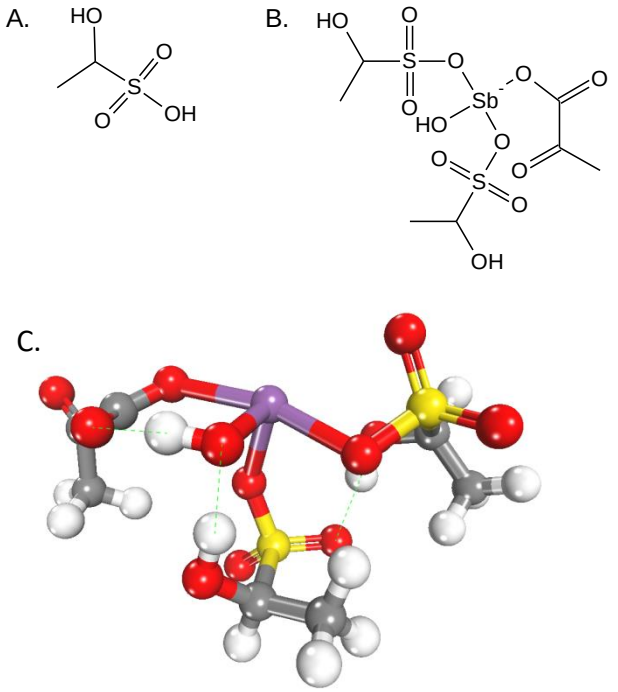

Figure 5. A: Possible reaction product formed from sulphites and ethanol. B: Proposed unknown Sb complex present in *raki* derived from high resolution and tandem mass spectrometry data. C: Ball and Stick representation of the geometry of the Sb complex as calculated by DFT. Sb, S, O, C and H atoms are represented as purple, yellow, red, grey and white spheres, respectively.

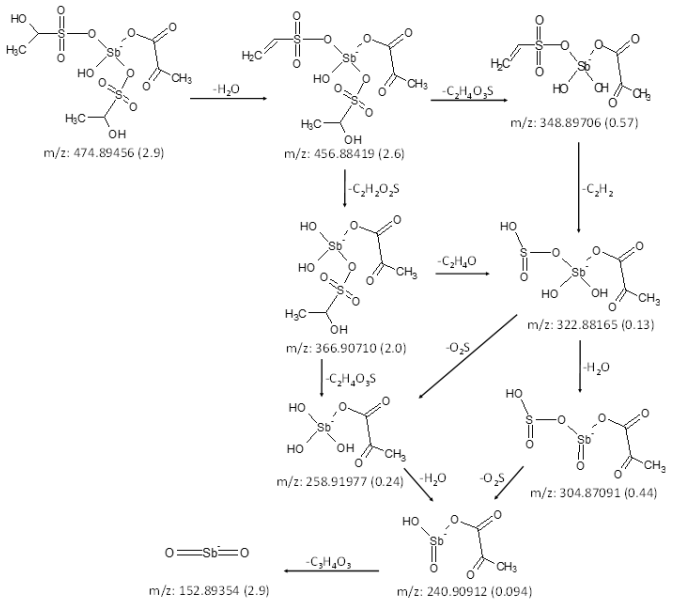

Figure 6. Proposed fragmentation route observed for the novel Sb complex present in *raki*. Accurate masses are given below each suggested structure along with mass accuracy in ppm in parentheses.

detected during MS^5 under any of the tested collision energies. During the tandem mass spectrometric analysis a mass loss of 108 was observed during MS^2, from ion 367-369 to ion 259-261; and a mass loss of 88 in MS^4 from ion 241-243 to ion 153-155.

After the CID fragmentation of the unknown Sb complex, the mass losses were studied and compared to the possible compounds present in the *raki* matrix. Tandem mass spectrometry by high resolution MS using a LTQ-ESI-Orbitrap was used to determine the exact mass of the compound and the product ions obtained. This allowed for determination of the elemental composition of the obtained ions and the composition of the mass losses.

In high resolution MS the R1 sample was analysed by direct infusion. As this operation mode provides less sensitivity than the LC-SSI-MS, used in the previous section, it was decided to spike the sample R1 at a concentration of 100 µg L^{-1}. Full mass spectra showed the same masses as previously described for low resolution ion trap mass spectrometry, i.e. 366.90710; 456.88419; and 474.89456.

After this, tandem mass spectrometry was carried out on these Sb-containing ions in order to obtain the exact mass of all the resulting product ions. For the m/z 366.90710, the same product ion formation route, as observed using the low resolution ion trap technique, was obtained. The m/z 456.88419 fragmented to the 366.90710 and to another one with the Sb isotopic distribution profile: 348.89706. The m/z 474.89456 fragmented to the 456.88419. Figure S1 (Supplementary material) depicts the most relevant tandem mass spectra obtained in this experiment.

These results demonstrate that ions with m/z 366.90710 and 456.88419 resulted from the precursor ion at m/z 474.89456 in addition to appearing in the full MS spectrum as well. According to this, the elemental composition of the ion 474.89456 and the fragments obtained was determined using appropriate software (*Thermo Xcalibur Qual Browser*). First, the program provided more than 10 possible molecular formulas for the target masses, with an error lower than 5 ppm. However, after analysing the obtained fragments in the different spectra, only two of the proposals were feasible: $C_7H_{14}O_{12}S_2Sb$ and $C_8H_{18}O_7S_4Sb$, with an error of 2.9 and 4.7 ppm, respectively. It is thought that the antimony complex present in *raki* corresponds to the first formula, as it presents a lower error and contains fewer sulphur atoms. The isotopic distribution was simulated so as to verify the proposed formulas by the software, obtaining good agreement.

After obtaining the elemental composition, the assignment of the ions obtained in the full MS and the tandem MS was performed. Table 1 summarises the m/z obtained with the typical isotopic distribution of the antimony and the molecular formulas obtained, together with the corresponding product ions and losses.

It was observed that the most significant mass losses were 108 and 88. Checking the results obtained, these losses correspond to the following formulas: $C_2H_4O_3S$ and $C_3H_4O_3$. As described in the literature, sulphites are commonly used as an additive for several foodstuffs and beverages, such as grapes, wines or beer.[29,30] Thus, sulphites may be added during the production of the *raki* and may have reacted with acetaldehyde (resulting from ethanol oxidation), forming the corresponding acetaldehyde bisulphite adduct (Figure 5A).[31,32] The second molecular formula may correspond to pyruvic acid, a product of alcoholic fermentation.[33] According to these results, the unknown Sb compound present in *raki* with a mass of 475 may

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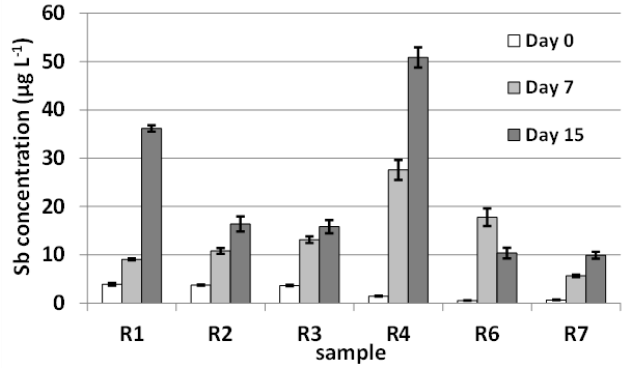

Figure 5. Evolution of total Sb concentration in PET bottled *raki* stored at 60ºC for 15 days.

be the complex depicted in Figure 5B. According to this structure and the mass losses observed, a proposed fragmentation route, from the precursor ion (m/z 474.89456) to the last product (m/z 152.89632) is proposed in Figure 6. The agreement of the measured product ion mass with the proposed structures are given in brackets as ppm values.

Theoretical calculations for proposed unknown Sb complex

Theoretical optimizations of the geometries of proposed molecular models showed that most of complexes had a tendency to dissociate with respect to their initial geometry and their initial chemical composition of the ligands that were used for each of the models. We were able to identify the most stable Sb complex with molecular formula $[Sb(C_2H_5O_4S)_2(C_3O_3H_3)(OH)]^-$ (Figure 5C) which is in agreement with the structure derived from the high resolution tandem mass spectrometric analysis. The geometry that can be seen in Fig. 5C corresponds also to the lower in energy conformer that was tested with the same molecular formula mentioned above. The geometry of the complex resembles a distorted pyramidal geometry which is further stabilized by the formation of three hydrogen bonds (ranging from 1.66 Å to 1.69 Å, shown in Fig. 5C with green dashed lines) between hydroxyl anion and the closest oxygen atom of the pyruvic anion, the hydroxyl group of hydroxyethanesulfonic anion with the hydroxyl anion, and the hydroxyl group of one hydroxyethanesulfonic anion with an oxygen atom of the other hydroxyethanesulfonic anion.

Antimony migration into *raki*

In this part of the study, the influence of storage time and temperature on Sb migration in *raki*, was investigated for the six PET bottled *raki* samples mentioned in section 2.3 (R1 – R4, R6 – R7). The effect of storage time up to 2 weeks at a storage temperature of 60 ºC was investigated. Both total Sb and speciation analysis was carried out by ICP-MS and LC-ICP-MS, respectively. The Sb content was determined at the beginning of the experiment, after 7 and 15 days of storage.

Total Sb

Total antimony concentrations throughout the experiment are plotted in Figure 7. *Raki* samples at the beginning of the test (day 0) showed significant concentrations of total Sb (0.5 – 3.9 µg L^{-1}). These values are comparable to those obtained in section 3.1 for the same samples. After a week of storage at 60ºC, *raki* samples showed a considerable increase in Sb concentration, exceeding the maximum level established by the EU for Sb in drinking waters. Sample R4 showed the highest concentration, reaching a value of 28 µg L^{-1}. After 2 weeks of storage, Sb concentrations keep increasing sharply in samples R1 and R4 reaching values of 36 and 51 µg L^{-1}, respectively, whereas in the rest of the samples this increase was not as pronounced, or even the values obtained were of the same order of magnitude as those obtained after 1 week.

Sb speciation

The analysis of Sb species in *raki* samples during the whole experiment are depicted in Figure 8. Concentrations of the organic complex of Sb were calculated by means of the external curve calibration of Sb(V) standards. At the beginning of the experiment, the predominant species was the proposed acetaldehyde-bisulphite pyruvate Sb complex in all the *raki* samples. However, after a week of storage, the concentration of this species decreased slightly or even disappeared as in the case of the R2 sample. Sb(V) concentration increased slightly in all samples (0.2 – 2.1 µg L^{-1}) whereas that of Sb(III) increased sharply (2.9 – 11.6 µg L^{-1}). After 2 weeks of storage, the presence of the organic complex of Sb kept decreasing and disappeared in samples R1, R3 and R4. Sb(V) concentration, with respect to the values obtained after 1 week of storage, increased slightly (0.8 – 26 µg L^{-1}) whereas Sb(III) had no specific trend. Sample R4 showed the highest concentration of Sb(III), reaching a value of 22 µg L^{-1}.

This fact could be due to a simultaneous release of antimony from PET and a conversion of the Sb species. *Raki* samples contain high amounts of allyl alcohols and other organic substances which can stabilise or even complex the Sb released from PET, as demonstrated in section 3.2. However, the exposure at high temperature may decompose these complexes to their inorganic species, and simultaneously, more Sb(III) may be released from PET. As an example,

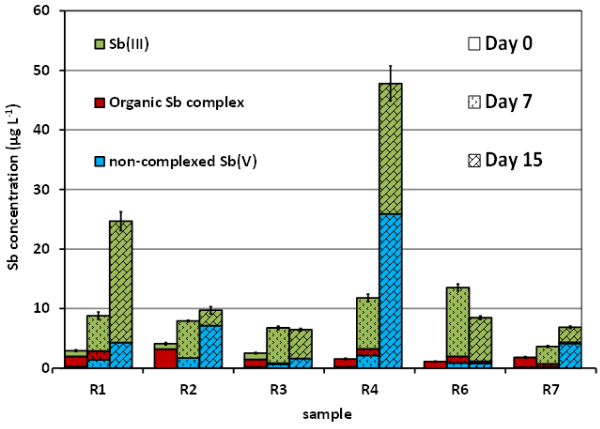

Figure 6. Evolution of Sb species concentrations in PET bottled raki stored at 60ºC for 15 days.

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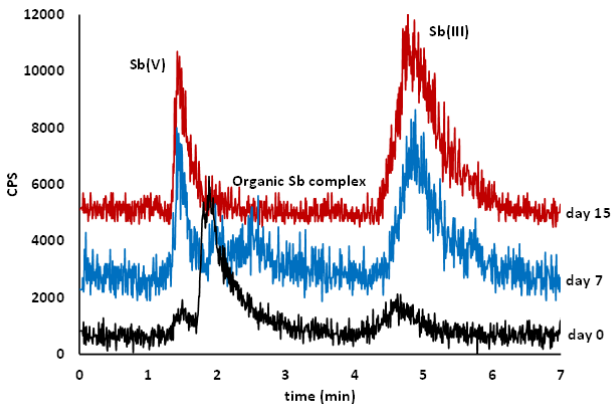

Figure 7. LC-ICP-MS chromatograms obtained for R1 at days 0, 7, and 15.

chromatograms obtained from the *raki* sample R1 are shown in Figure 9.

Table 2 summarises the total Sb concentration and the sum of the species obtained together with the recovery. As observed in section 3.2, good recoveries, from 70 to 110%, were observed in some cases. However some low recoveries were also observed (down to 40%). These facts demonstrate again that some other minor Sb species could be present in the *raki* samples but are not detected as they do not elute from the column.

These high Sb concentrations obtained in the migration experiment exceed the maximum limit of the total antimony in water (5 µg L^{-1}) and are much higher than the Sb concentrations obtained in the high temperature migration studies previously published,[19] in which concentrations up to 6 µg L^{-1} were reached at 15 days of storage in PET bottled waters. Moreover, the more toxic Sb(III) species predominant in some of the samples. Thus, it is recommended not to expose spirit beverages to elevated temperatures if they are stored in PET, as their matrix characteristics may easily promote Sb migration from PET.

Conclusions

Reliable and reproducible determination of Sb in spirit samples has been assessed by ICP-MS. Concentrations up to 4 µg L^{-1} were found, which is close to the maximum limit established by the EU in drinking waters. The most suitable LC procedure for Sb species separation consisted of using as mobile EDTA 10 mmol L^{-1} at pH 4 with 0.5% methanol. The predominant Sb species is an organic Sb complex. This complex was identified by LC-SSI- and ESI- MS, whose structural elucidation was established using HRMS. The proposed structure resulted in a complex with m/z 474.89456 and the molecular formula $[Sb(C_2H_5O_4S)_2(C_3H_3O_3)(OH)]^-$, taking as ligands one molecule of pyruvic acid and two molecules of an acetaldehyde-bisulphite adduct.

According to the conducted migration studies, temperature and spirit matrix potentially affects Sb leaching from PET bottles, producing a rapid release in just one week of storage. Extremely high concentrations up to 51 µg L^{-1} were obtained. It is therefore highly recommended not to expose PET bottled beverages to elevated temperatures, as a rapid release of Sb has been demonstrated. The results obtained in this study could be used in the proposal of further Directives on Sb in other beverages other than from water.

Table 2. Total Sb concentration and sum of the species (µg L^{-1}) together with the column recovery for PET bottled *raki* samples stored at 60ºC for 15 days.

Day	Sample	Total Sb	Sum of species	Column recovery (%)
0	R1	3.91 ± 0.28	2.94 ± 0.13	75.3
	R2	3.73 ± 0.12	4.12 ± 0.20	110.4
	R3	3.64 ± 0.16	2.53 ± 0.13	69.5
	R4	1.45 ± 0.09	1.55 ± 0.11	107.5
	R6	0.52 ± 0.02	1.124 ± 0.073	216.5
	R7	0.66 ± 0.04	1.80 ± 0.15	272.4
7	R1	9.07 ± 0.21	8.81 ± 0.62	97.2
	R2	10.79 ± 0.60	7.92 ± 0.12	73.4
	R3	13.13 ± 0.68	6.77 ± 0.28	51.5
	R4	27.57 ± 2.07	11.79 ± 0.62	42.8
	R6	17.77 ± 1.81	13.54 ± 0.57	76.2
	R7	5.63 ± 0.23	3.63 ± 0.17	55.4
15	R1	36.16 ± 0.65	24.68 ± 1.58	68.3
	R2	13.37 ± 1.56	9.72 ± 0.61	59.4
	R3	15.83 ± 1.35	6.46 ± 0.21	40.8
	R4	50.85 ± 2.09	47.79 ± 2.93	94.0
	R6	10.36 ± 1.09	8.46 ± 0.31	81.7
	R7	9.92 ± 0.71	6.87 ± 0.20	69.3

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