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CYCLE XXXI

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PhD Course

QUÍMICA FINA

University of Córdoba

DETERMINATION OF XENOBIOTICS AND CONCENTRATION LEVELS OF ENDOGENOUS SUBSTANCES IN BIOLOGICAL MATRICES

DETERMINACIÓN DE XENOBIÓTICOS Y CONCENTRACIÓN DE SUSTANCIAS ENDÓGENAS EN MATRICES BIOLÓGICAS

SUPERVISORS:
GIANPIERO BOATTO
SOLEDAD RUBIO BRAVO
DIEGO GARCÍA GÓMEZ

FRANCESCA ACCIONI

Córdoba 12/12/2018

TITULO: *DETERMINATION OF XENOBIOTICS AND CONCENTRATION LEVELS OF ENDOGENOUS SUBSTANCES IN BIOLOGICAL MATRICES*

AUTOR: *Francesca Accioni*

Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

https://www.uco.es/ucopress/index.php/es/
ucopress@uco.es

Tesis Doctoral:

Determination of xenobiotics and concentration levels of endogenous substances in biological matrices

Trabajo presentado, para optar al grado de doctor, por

Francesca Accioni

que lo firma en Córdoba, a 12 de diciembre de 2018

Firmado:

Francesca Accioni

Licenciada en Química y tecnologías farmacéuticas (Universidad de Sassari, Italia)

con el VºBº de los directores

Firmado:

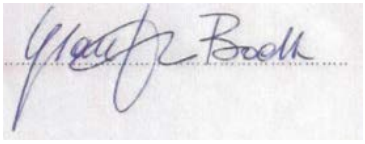
Soledad Rubio Bravo

Catedrática de Química Analítica de la Universidad de Córdoba

Firmado:

Diego García Gómez

Profesor Ayudante Doctor de Química Analítica de la Universidad de Salamanca



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CERTIFICAN: Que la citada Tesis Doctoral *"Determination of xenobiotics and concentration levels of endogenous substances in biological matrices"* se ha realizado en los laboratorios del Departamento de Química Analítica de la Universidad de Córdoba y del Departamento de Química y Farmacia de la Universidad de Sassari y que, a su juicio, reúne todos los requisitos exigidos a este tipo de trabajos.

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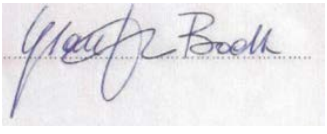

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Profesor titular del de Departamento de Química y Farmacia de la Universidad de Sassari

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And last but not least, I thank my friends Maria, Maria Grazia, Noelia, Ana, Sole, Encarni, Salatti, Laura and Rocío for their help and for all the fun we have had in the last three years.

TÍTULO DE LA TESIS: Determination of xenobiotics and concentration levels of endogenous substances in biological matrices

DOCTORANDO/A: FRANCESCA ACCIONI

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

Las investigaciones desarrolladas en esta Tesis Doctoral, realizada en régimen de cotutela entre la Universidad de Cagliari-Sassari (Italia) y la Universidad de Córdoba (España) han tenido como objeto la simplificación del tratamiento de muestras biológicas para la determinación de compuestos xenobióticos y sustancias endógenas mediante cromatografía de líquidos acoplada a diferentes detectores (espectrometría de masas y ultravioleta). Para ello se han utilizado dos estrategias; precipitación con proteínas mediante acetonitrilo (PPT-ACN) y extracción-eliminación de interferencias con el uso de disolventes supramoleculares (SUPRAS).

La primera estrategia (PPT-ACN) se ha aplicado al desarrollo de métodos simples basados en LC-UV para la determinación de antioxidantes endógenos, concretamente β-carotene, retinol y/o α-tocopherol, en suero de caballos de raza. Las metodologías desarrolladas han tenido como finalidad la determinación de la concentración basal del antioxidante, el establecimiento de correlaciones entre la concentración en suero del antioxidante investigado y diferentes aspectos biológicos tales como fotoprotección o calidad del semen, o su posible utilización como marcadores del tipo de alimentación.

La segunda estrategia basada en la combinación de SUPRAS y LC-MS/MS se ha aplicado al desarrollo de metodologías selectivas para la determinación de multicomponentes (compuestos xenobióticos y endógenos) en matrices biológicas. Para ello se han utilizado SUPRAS volátiles con propiedades de acceso restringido basados en hexanol y se han sintetizado nuevos SUPRAS a partir del ácido heptafluorobutírico. Estas metodologías han permitido la determinación de anfetaminas en una amplia variedad de matrices (saliva, orina, suero, sudor, leche humana, pelo y uñas) y antihelmínticos en leche de vaca. Se ha demostrado que el SUPRAS basado en ácido heptafluorobutírico tiene elevada capacidad de extracción para compuestos orgánicos catiónicos de elevada polaridad tales como amino ácido y oligopéptidos, lo que ha permitido su aplicación a la determinación de opiorfina en saliva humana.

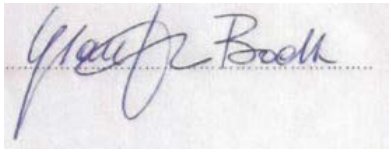
Los resultados de las investigaciones realizadas se han materializado en 7 artículos científicos (3 publicados en Talanta (Q1), Journal of Agricultural and Food Chemistry (Q1), Ecology and Evolution (Q2)) y 4 enviados para su publicación en revistas científicas indexadas). Los resultados obtenidos se han presentado por la doctorando en 3 contribuciones orales a congresos (3 internacionales) y 3 carteles (2 nacionales y 1 internacionales).

En base a la originalidad de las investigaciones desarrolladas y expuestas en esta Memoria así como la excelente formación científica adquirida por Dña. Francesca Accioni, autorizamos la presentación de esta Tesis Doctoral.

Córdoba, 12__ de __diciembre__ de ___2018___

Firma del/de los director/es

Fdo.:___Diego García Gómez_____ Fdo.: __Soledad Rubio Bravo_



Fdo.: Gianpiero Boatto

A mio marito Giovanni e ai miei genitori che sempre hanno creduto in me e mi hanno sostenuta in tutte le mie scelte...

...a Litz che mi ha strappato un sorriso anche quando non avevo tanta voglia di farlo.

INDEX

TITLE: DETERMINATION OF XENOBIOTICS AND CONCENTRATION LEVELS OF ENDOGENOUS SUBSTANCES IN BIOLOGICAL MATRICES

INDEX:

Aim:

Simplification and reduction of sample treatment are very important aspects today in analytical chemistry, especially considering the social demand for analysing complex matrices and developing a greener chemistry. Two issues need to be particularly fulfilled: reducing the amount of organic solvents employed in analytical processes and the duration of the analysis. These purposes are in line with the principles of green analytical chemistry (GAC), and with European policy, which leads to minimize emissions of volatile organic compounds (VOCs); in fact, it is estimated that 25% of VOC pollution in Europe arises from the use of organic solvent. Moreover, the optimization of the analysis time is a key point to cut costs and to allow an easier transfer to routine applications.

Over the last decade, solid-phase micro-extraction, miniaturization, auxiliary energies, and novel solvents (e.g., supercritical fluids and ionic liquids) have been proposed as alternatives to classical approaches. On this field, the use of supramolecular solvents (SUPRAS), which consist of nanostructured liquids generated from the self-assembly of amphiphilic compounds, is gaining momentum. SUPRAS, whose synthesis is a spontaneous and tailored process which depends on environmental conditions, have been shown, during the last years, to offer a great potential for reducing and simplifying sample treatment processes.

Taking into consideration all these premises, the aim of this thesis is the development of new analytical methodologies aiming to detect xenobiotics and endogenous substances in complex biological matrices, focusing on simplifying the sample treatment by reducing organic solvent consumption and looking for providing new tools for veterinary, forensic, clinical and food quality control purposes.

In order to accomplish this aim, protein precipitation (PPT) with acetonitrile (ACN), and SUPRAS-based extraction, were proposed as sample treatment techniques.

In this context, the specific objectives of this Thesis have been as follows:

1) Explore the use of PPT and SUPRAS for the determination of xenobiotics and endogenous substances in several different biological matrices (saliva, urine, plasma, milk, sweat, hair and fingernail).

2) Develop analytical methodologies based on PPT and SUPRAS that may be able to resolve real bioanalytical problems by means of their combination with liquid chromatography coupled to UV-visible and tandem mass spectrometry detectors.

At the same time, a fundamental aim in this Thesis has been the development of a Formation Program for the PhD student under a joint supervision at the Universities of Cagliari and Sassari (Italy), and the University of Córdoba (Spain). This Program included the attendance and presentation of contributions to international congresses, the writing of scientific papers, teaching of BSC students, and the co-direction of BSC theses.

Content:

The content of this PhD Thesis is divided in two parts preceded by an Introduction, where the need to develop new strategies for sample treatment, the analytical technique of protein precipitation in biological matrices, and the theoretical and practical aspects of SUPRAS are discussed.

The content of the two parts are as follows:

Part A: Determination of air and light sensitive endogenous antioxidants in serum by protein precipitation and high-performance liquid chromatography.

In this part, four scientific papers are reported (chapters 1-4). They are all related to the use of protein precipitation (PPT) based on denaturisation by the addition of acetonitrile to samples that are then just vortex-shaken and centrifuged. The supernatant obtained is finally dried under a stream of nitrogen and injected into high-performance liquid chromatography with ultraviolet detection (HPLC- UV). In all cases, the method was fully validated following international guidelines. In Chapter 1, potential intra- and inter-specimen fluctuations of β-carotene and retinol are monitored in serum of Asinara (albino) and Sardo (grey) breed donkeys, in order to rationalize an alternative metabolic pathway to explain photoprotection. In Chapter 2, concentrations of α-tocopherol in serum of free ranging Asinara (albino) vs. Sardo (grey) donkeys are evaluated as potential markers of natural feedstuff selection. In Chapter 3, the α-tocopherol content in serum of stallions under a supplemented diet is monitored with the goal to prove a positive correlation between the antioxidant levels in these animals and semen quality. Finally, the importance to have an effective and simply tool for the determination of α-tocopherol in serum is also supported in Chapter 4, where baseline levels of this circulating antioxidant in free ranging and confined Giara horses were evaluated.

Part B: SUPRAS-based matrix-independent platforms for quantifying multi compounds in biological matrices by LC-MS/MS for forensic, clinical and food quality control purposes.

The content of the second part of this Thesis (chapters 5-7) is focused on the development of analytical methods based on SUPRASs, which integrate extraction and matrix clean-up. In Chapter 5, a universal sample treatment for simplifying the determination of amphetamine-type stimulants (ATS) in oral fluid, urine, serum, sweat, breast milk, hair and fingernails is reported. Hexanol based SUPRAS were synthetized *in situ* and the extract was directly

injected into LC-MS/MS. In chapter 6, a new SUPRAS, based on a perfluorinated amphiphile, was investigated for the extraction of oligopeptides and aminoacids from aqueous matrices. This novel SUPRAS was subsequently applied for the determination of opiorphin levels in saliva by the direct injection of the SUPRAS into the LC-MS/MS system. In Chapter 7, a fast and reliable method for the determination of anthelmintic benzimidazoles in milk by LC-MS/MS is proposed. For this purpose, a hexanol-based SUPRAS with restricted access and volatile properties was applied. It should be highlighted that all the analytical methods described in this part were fully validated following the appropriate international guidelines.

RESUMEN DE LA TESIS DOCTORAL DE Dª FRANCESCA ACCIONI.

1. Introducción o motivación de la tesis

Esta Tesis Doctoral ha tenido como objeto de estudio el desarrollo y aplicación de nuevos y eficientes procesos de extracción de xenobioticos y substancias endógenas en matrices biológicas complejas, basados en química verde [1, 2]. Los métodos que se han desarrollado han pretendido extender la utilización tanto de la precipitación de proteínas séricas con acetonitrilo (PPT) [3] como de los disolventes supramoleculares (SUPRAS) [4, 5] a aplicaciones veterinarias, forenses, clínicas y de seguridad y calidad alimentaria.

2. Contenido

La Memoria de esta Tesis Doctoral se ha estructurado en dos bloques, precedidos de una Introducción en la que se describen las nuevas estrategias de la Química Bioanalítica para la reducción del consumo de disolventes orgánicos en procesos de extracción analítica de substancias endógenas y xenobióticos en matrices biológicas. Asimismo, se ilustran los aspectos teóricos y prácticos de las dos metodologías extractivas aplicadas en esta Tesis (precipitación de proteínas con acetonitrilo y SUPRAS). Los contenidos de los dos bloques se especifican a continuación:

Bloque A: Determinación de antioxidantes endógenos, con alta sensibilidad a la luz y al aire, a través una metodología basada en tratamiento de muestras con precipitación de proteínas y cromatografía de líquidos con detección UV.

En este bloque se describe una metodología analítica para la determinación de β-caroteno, retinol y α-tocoferol en suero de burros y caballos y cuatro de sus diversas aplicaciones en clínica veterinaria (capítulos 1, 2, 3, 4). Este tratamiento de muestra consistió en la adición de 600 microlitros de acetonitrilo a 300 microlitros de suero, lo que resultó en la precipitación de las proteínas. El extracto así obtenido se secó y redisolvió en 150 microlitros de fase móvil para su posterior análisis mediante cromatografía de líquidos con detección UV. El método fue validado cumpliendo todos los requisitos internacionales requeridos.

Bloque B: Plataformas analíticas independientes de la matriz y basadas en SUPRAS para la cuantificación de multicomponentes en multimatrices mediante cromatografía de líquidos acoplada a espectrometría de masas en tándem.

El objeto de las investigaciones presentadas en este bloque ha sido el desarrollo de metodologías innovadoras, basadas en SUPRAS, para la determinación de anfetaminas (capítulo 5), aminoácidos y oligopéptidos (capítulo 6) y antihelmínticos (capítulo 7) en muy diversas matrices biológicas. Como tratamiento de muestra se utilizaron dos SUPRAS diferentes: uno obtenido mediante la coacervación de disoluciones coloidales de micelas inversas de hexanol (capítulos 5 y 7), y un segundo formado por coacervación de ácido heptafluorobutírico (HFBA) en disoluciones acuosas ácidas (capítulo 6). Los dos SUPRAS estudiados presentaron propiedades de acceso restringido. En ambos casos se desarrollaron procedimientos generalizados que demostraron ser independientes de la matriz, y capaces de simplificar en una sola etapa todo el proceso de purificación y extracción (<35 minutos). Las matrices biológicas estudiadas fueron orina, suero, saliva, sudor, leche materna, pelo, uñas y leche de vaca para consumo humano. Todos los extractos obtenidos fueron analizados mediante cromatografía de líquidos acoplada a espectrometría de masas en tándem. Los tres métodos desarrollados fueron totalmente validados según la legislación internacional pertinente.

3. Conclusiones

Las principales conclusiones que pueden extraerse de esta tesis son:

Bloque A: Determinación de antioxidantes endógenos, con alta sensibilidad a la luz y al aire, a través una metodología basada en tratamiento de muestras con precipitación de proteínas y cromatografía de líquidos con detección UV.

- La metodología analítica basada en el tratamiento de muestra mediante la precipitación de proteínas (PPT) con acetonitrilo y posterior análisis mediante cromatografía de líquidos con detección UV ha demostrado una excelente capacidad para la determinación de antioxidantes endógenos de elevado coeficiente de partición (XLogP3 13.5, 10.7 and 5.7 para β-caroteno, α-tocoferol y retinol, respectivamente). Además, se han obtenido recuperaciones cuantitativas (70-120%) y óptimos límites de detección (0.036 $\mu g\ mL^{-1}$), gracias al factor de pre-concentración alcanzado (x2).

- PPT ha demostrado ser una metodología verde, adecuada para el tratamiento de muestras de suero. Además, la inducción de precipitación de proteínas (>90%) mediante una relación muestra/ACN de 1:2, la posterior evaporación de la disolución resultante hasta sequedad, y la redisolución del extracto en fase móvil (relación

muestra/solución de reconstitución de 2:1), han resultado en la eliminación del efecto matriz y garantizado el empleo de poco disolvente, el aumento del factor de pre-concentración y una óptima compatibilidad con el sistema UV.

- El método analítico desarrollado y empleado en los capítulos 1-4 ha sido totalmente validado siguiendo las líneas guías internacionales, obteniéndose valores óptimos de linealidad, sensibilidad, precisión y exactitud.

Bloque B: Plataformas analíticas independientes de la matriz y basadas en SUPRAS para la cuantificación de multicomponentes en multimatrices mediante cromatografía de líquidos acoplada a espectrometría de masas en tándem.

- La extracción mediante SUPRAS basados en hexanol ha permitido obtener extracciones eficientes para analitos (anfetaminas y antihelmínticos) con características químico-físicas muy diferentes, gracias a la capacidad del SUPRAS de disolver solutos mediante un mecanismo mixto (interacciones de van der Waals, puentes de hidrógeno, interacciones polares, e interacciones π-catión). Además, estos SUPRAS han demostrado tener una aplicabilidad excelente (con eliminación de los interferentes endógenos) para numerosas matrices biológicas (suero, orina, saliva, sudor, leche materna, leche de vaca, pelo y uñas). Estos SUPRAS han demostrado ser disolventes "à la carte" que se pueden sintetizar de forma totalmente espontánea y directa en matrices liquidas. Además, tienen capacidad para actuar como materiales con propiedades de acceso restringido (RAM), o como agentes volátiles con propiedades de acceso restringido (RAM-VOL-SUPRAS) si se añade al proceso una etapa de evaporación para la eliminación de los fosfolípidos. Gracias a esta propiedad, las moléculas de bajo peso molecular se solubilizan en el SUPRAS, mientras que interferentes como proteínas o polisacáridos están excluidos física (precipitación por THF) y/o químicamente (fenómenos de exclusión). Estos SUPRAS basados en hexanol han permitido la determinación de 5 anfetaminas en 7 matrices biológicas diferentes y 8 benzimidazoles antihelmínticos en leche de vaca mediante cromatografía de líquidos acoplada a espectrometría de masas en tándem (LC-MS/MS). Los límites de detección encontrados están, para todos los casos, por debajo de los límites legislados y pertinentes para cada analito.

- Se ha propuesto en esta Tesis, por primera vez, un SUPRAS basado en ácido heptafluorobutírico (HFBA) como disolvente alternativo para la extracción de analitos muy polares (aminoácidos y oligopéptidos), cuya extracción en muestras biológicas se considera una tarea muy compleja. Esta interesante capacidad de extracción está relacionada con la "polaridad hidrofóbica" que presentan los compuestos perfluorados. Así, este SUPRAS ha sido capaz extraer de con elevado rendimiento (recuperaciones>80%) 20 aminoácidos y 9 oligopéptidos con valores de D de hasta -3. Adicionalmente, se ha llevado a cabo también la determinación del oligopéptido Opiorfina en saliva, por inyección directa en LC-MS/MS del extracto del SUPRAS basado en HFBA.

- Las metodologías analíticas de extracción/limpieza basadas en SUPRAS constan de una única etapa que permite tratar muestras de forma simple, en muy poco tiempo, y empleando poca cantidad de muestra y de disolventes orgánicos (tiempo 15-35 min, muestra ≤ 1 mL, disolvente orgánico <2 mL). Por estos motivos, estas metodologías basadas en SUPRAS pueden encuadrarse dentro de la química analítica verde.

- Las tres metodologías basadas en SUPRAS han sido totalmente validadas siguiendo los parámetros requeridos por las respectivas guías internacionales, obteniéndose valores óptimos de linealidad, sensibilidad, selectividad, precisión y exactitud.

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INTRODUCTION

1. Bioanalytical chemistry: determination of xenobiotics and endogenous substances

Bioanalytical chemistry is considered a sub-discipline of analytical chemistry, which includes the qualitative and quantitative analysis in biological matrices of all the compounds particularly involved with life and health processes (i.e. xenobiotics and endogenous substances). The word xenobiotic comes from the Greek *xenos*, meaning guest, friend, or foreigner. Xenobiotics can be defined as "any foreign substances or exogenous chemicals which the body does not recognize, such as drugs, pollutants, as well as some food additives and cosmetics" [1]. Or in a similar definition: "xenobiotics are chemicals found in but not produced by organisms or the environment. Some naturally occurring chemicals (endobiotics) become xenobiotics when present in the environment at excessive concentrations" [2]. On the other hand, endogenous substances are compounds that originate from within an organism; with their unnatural location or concentration reflecting the health status of the organism [3]. Taking into account the large number of analytes and biological matrices involved in this area, bioanalysis has taken an important role in fields such as biological sciences, health sciences, earth and environmental sciences, and physical sciences.

Nowadays, as well as other areas of analytical chemistry, bioanalytical chemistry focuses on the development of new green methodologies to provide fast and very sensitive quantitative analyses, useful as routine laboratory techniques. Requirements for such effective strategies are linked to the increasing number of biological samples and to the decreasing target concentrations [4].

Bioanalytical methods usually involve several steps (Figure 1): sample collection, extraction and matrix clean-up procedures, chromatographic analysis and detection [1, 4].

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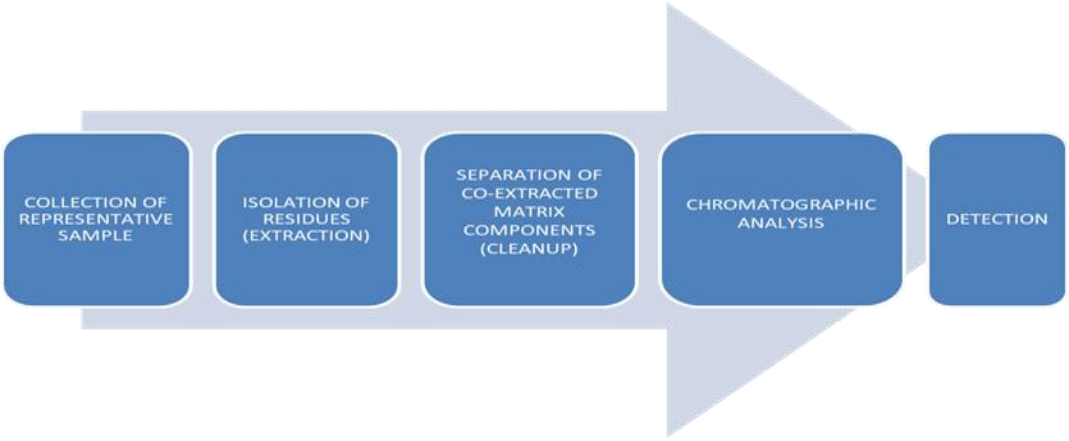

Figure 1. Schematic representation of a bioanalytical method.

1.1. New sample treatments in bioanalytical chemistry: strategies to reduce the use of organic solvents

During the last years, researchers have developed new reliable analytical strategies in order to reach a compromise between the performance parameters of the method and their impact on the environment [5]. Green analytical chemistry (GAC) appeared in 2000 [6] and its main principles are:

- elimination or reduction of the use of chemical products (e.g., organic solvents)
- reduction of energy consumption
- reduction of waste
- safety for the analyst.

Furthermore, European policies lead to minimize emissions of volatile organic compounds (VOCs) providing guidance documents on the use of chemicals and methods which affect the environment and human health [7].

Because of the complex composition of biological matrices, sample treatment is the most important step aiming to provide the target analytes at a properly concentration for further determination and to reduce or eliminate the matrix effect coming from interferences. In fact, the capability of the technique to concentrate the analyte in the original biological matrix allows an increase in sensitivity that results in the improvement of the limits of quantification of the method. Moreover, matrix manipulation allows removing endogenous

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[6] A. Galuszka, Z. Migaszewski, J. Namieśnik, Trac.-Trend Anal. Chem., 50 (2013) 78–84.
[7] http://ec.europa.eu/environment/archives/air/stationary/solvents/exchange.htm (last access 23 July 2018).

compounds which co-eluate with the analyte such as proteins, which affect the chromatographic process, and phospholipids, which cause ion suppression (loss of signal) in LC/MS [8]. Sample preparation in bioanalysis is commonly carried out by liquid-liquid extraction (LLE) and solid phase extraction (SPE) [1, 8].

One of the alternative sample treatments recently developed are QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe), protein precipitation (PPT), supercritical fluids, ionic liquids and supramolecular solvents (SUPRAS) combined with miniaturized procedures [9, 10, 11, 12, 13]. All of them, in one way or another, are considered green approaches.

LLE is based on differences in solubility (octanol-water partition coefficient), being the analyte extracted by a partitioning mechanism [14]. In the case of liquid biological matrices (e.g., plasma), a water-immiscible solvent is directly mixed with sample and, afterwards, usually centrifugation, evaporation and reconstitution with an instrument-compatible solvent of the extract are required [15]. It presents some drawbacks such as the use of high amounts of sample and organic solvents, which make LLE a time-consuming and "no-green" procedure. Anyway, LLE is still a highly used sample treatment in bioanalytical chemistry. To improve the throughput of the method, a semiautomatic 96-well plate format was developed by L. Ramos et al. in 2000 [16], although it presented complexities due to contamination problems [15]. Liquid phase microextraction (LPME) was introduced in 1996 by H.H. Liu et al. to minimize the generation of VOCs. It is a miniaturized liquid–liquid extraction that it is based on the same principle of LLE, but the analyte is extracted in a very small volume of organic solvent. SALLE is a subtype of LLE, where salts, such as magnesium sulfate, are added to the matrix prior to the mixing of the organic solvent. By the presence of the salt, the solvent is salted-out and it forms a separated phase, where the target analytes are

[8] R. Dams, M.A. Huestis, W.E. Lambert, C.M. Murphy, J. Am. Soc. Mass. Spectr., 14 (2003) 1290–1294.
[9] K.D. Clark, M.J. Trujillo-Rodríguez, J.L. Anderson, Anal. Bioanal. Chem., 410 (2018) 4567–4573.
[10] L.A. Blanchard, J.F. Brennecke, Ind. Eng. Chem. Res., 40 (2001) 287–292.
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[14] Z. Berk, Extraction. In: Z. Berk (eds) Food Process Engineering and Technology. Elsevier, (2013).
[15] Y.Q. Tang, N. Weng, Bioanalysis, 5 (2013) 1583–1598.
[16] L. Ramos, R. Bakhtiar, F.L. Tse, Rapid. Commun., Mass. Spectrom., 14 (2000) 740–745.

extracted. This technique allows to save sample, to reduce the whole amount of organic solvent, and provides good enough recoveries and limits of quantification [15, 17].

Ultrasonic assisted extraction (UAE) and microwave-assisted extraction (MAE) are also considered green methods based on LLE, but their application mainly involves solid or semisolid samples (replacing Soxhlet extraction). UAE employs acoustic vibrations which produce cavitation in the extraction solvent. Thus, there is an enhancement in the analyte extraction. Usually, filtration/centrifugation and exsiccation/reconstitution of the extract are required. The drawbacks of UAE are the no-uniformity of ultrasound vibrations and a very low selectivity. In MAE, the organic solvent is heated by the use of microwaves. The main advantage of MAE is its short duration and low consumption of solvent, but as well as UAE selectivity is very low [4].

Pressurized Liquid Extraction (PLE) [18] can also be considered a green sample treatment. It is based on solid/liquid extraction but applying high temperatures and pressures. It was introduced for the first time in 1995 as Accelerated Solvent Extraction Technology (ASE®). Authors consider this technique a valid alternative to Soxhlet and solid/liquid extraction because it provides similar recoveries and guarantees lower consumptions of time and solvents [18].

SPE is one of the most used techniques in fields such as environmental science, health science and foodstuff analysis. It allows to both: extract analytes from complexes matrices and clean-up the sample. The principle that rules the process is very similar to that of liquid/liquid extraction. It includes the partition of the analyte between the stationary absorbent phase and the liquid phase. It is possible to picture the whole process in fourth steps: conditioning, sample loading, washing and elution (Figure 2). The choice of solvent and stationary phase (polar phases, non-polar phases, ion exchange phases, immune-affinity SPE phases…) is the critical point of the method. Otherwise, the amount of organic solvents involved in the procedure is the key point in terms of VOC emissions [19, 20].

[17] C. Tejada-Casado, M. del Olmo-Iruela, A.M. García-Campaña, F.J. Lara, J. Chromatogr. B, 1091 (2018) 46–52.

[18] A. Mustafa, C. Turner, Anal. Chim. Acta, 703 (2011) 8–18.

[19] P. Lucci, D. Pacetti, O. Núñez, N.G. Frega, Current Trends in Sample Treatment Techniques for Environmental and Food Analysis. In: L. de Azevedo Calderon (eds) Chromatography - The most versatile method of chemical analysis. InTech, (2012).

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Solid-phase microextraction (SPME) was presented for the first time by Belardi and Pawliszyn in 1989, and it showed an advance in terms of VOC emission reduction and green chemistry. In this technique, there is a sorption of the target compounds directly from the matrix or in the headspace on a fused-silica fiber, while desorption is achieved thermally or by the use of solvents [21].

Stir-bar sorptive extraction (SBSE) was developed in 1999 by Baltussen et al. and its principle is based on the interaction of analytes with a film of polydimethylsiloxane (PDMS). The step of desorption consists in the use of a small quantity of solvent in case of no-volatile compounds or thermally, when analytes are volatile or semi-volatile [22]. On-line SPE also focuses on saving solvent consumption [20].

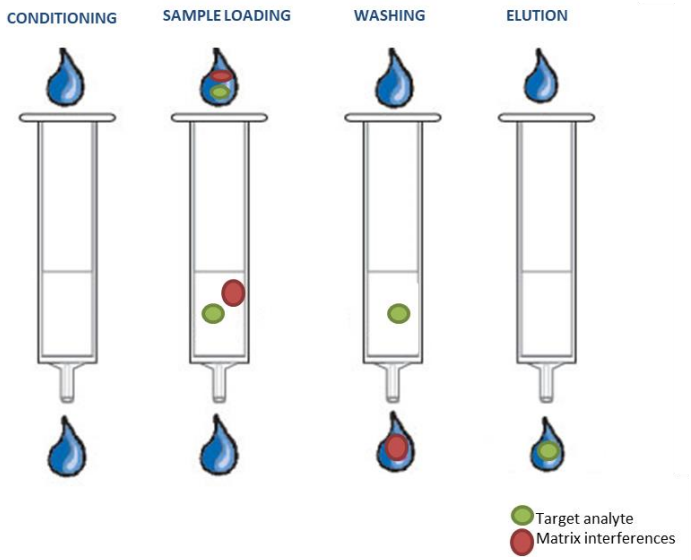

Figure 2. Scheme of the four step involves in classic solid-phase extraction (SPE).

The main advantages of all microextraction techniques (e.g. liquid-liquid microextraction, SPME, SBSE...) over LLE and SPE are minimal consumption of organic solvents, which decreases the negative impact of the procedures on the environment, and higher enrichment factors, which enhances the sensitivity [23].

QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) was developed by Anastassiades et al. in 2003 for the extraction of pesticides [24]. Since this moment, it has

[21] R.P. Belardi, J.B. Pawliszyn, Water Pollut. Res. J. Canada, 23 (1989) 179–191.
[22] E. Baltussen, P. Sandra, F. David, C.J. Cramers, Microcol. Sep., 11 (1999) 737–747.
[23] K. Ridgway, S.P. Lalljie, R.M. Smith, J. Chromatogr. A, 1153 (2007) 36–53.
[24] M. Anastassiades, S.J. Lehotay, D. Stajnbaher, F.J. Schenck, J. AOAC Int., 86 (2003) 412–31.

become a very popular sample treatment in bioanalysis, being applied to many different types of biological matrices and target compounds. The methodology is based on a combination of SALLE and SPE. Therefore, it can be divided in two steps: a liquid/liquid extraction, enabled by a salting-out process; and a cleaning-up which is carried out by dispersive SPE [25] (Figure 3).

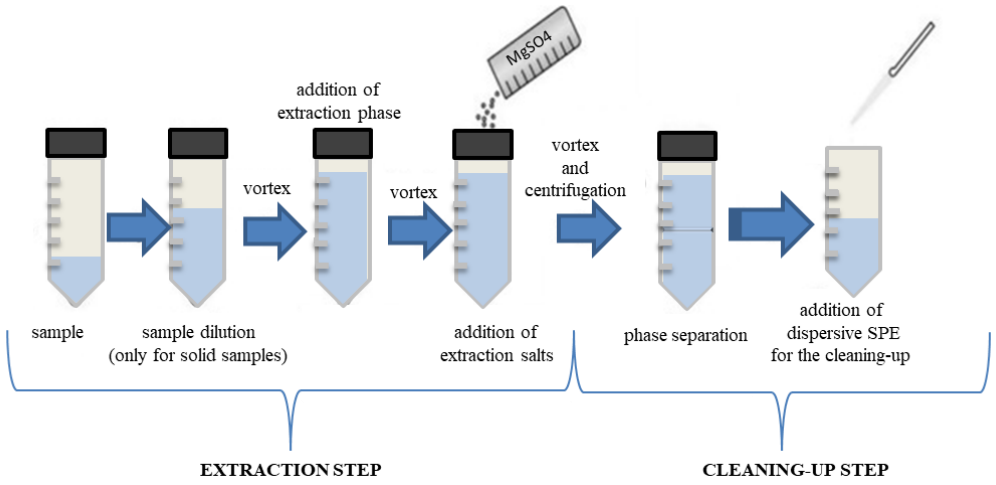

Figure 3. QuEChERS procedure as proposed by Anastassiades et al. [24].

In 2007, QuEChERS was regarded by the Association of Official Analytical Chemists (AOAC) as one of the best effective sample preparation techniques for pesticide residue analysis [26, 27]. It can be considered a green technique because it involves a minimal amount of sample and toxic solvents [26], and since 2003 to date, many modifications have been proposed to improve the throughput. The most used QuEChERS-dSPE kits are sold for foodstuff analysis and are divided into 4 categories: for general samples, for samples with colored extracts, for samples which contain waxes or fats, and for samples with fats and pigments [27]. Although at first QuEChERS method was developed as sample treatment for pesticides quantification in food, recently, it is becoming a common procedure also in bioanalytical fields such as forensic science, clinical science and environmental science [13, 28, 29].

[25] B. Łozowicka, E. Rutkowska, M. Jankowska, Environ. Sci. Pollut. R., 24 (2017) 7124–7138.
[26] J. Stocka, M. Tankiewicz, M. Biziuk, J. Namieśnik, Int. J. Mol. Sci., 12 (2011) 7785–7805.
[27] A. Lawal, R.C.S. Wong, G.H. Tan, L.B. Abdulra'uf, A.M.A. Alsharif, J. Chromatogr. Sci., 56 (2018) 656–669.
[28] A. Pouliopoulos, E. Tsakelidou, A. Krokos, H.G. Gika, G. Theodoridis, N. Raikos, J. Anal. Toxicol., 42 (2018) 337–345.

Supercritical fluid extraction (SFE) is a sample treatment of increasing interest in bioanalytical chemistry. The main field of application concerns drugs detection in human fluids. SFE is being employed in analytical chemistry since the late 80s. It is like Soxhlet extraction, but it involves solvents above their critical temperatures and pressures, i.e., supercritical fluids (SFs). CO_2 is the most used SF, because of its properties being non-toxic and cheap. This technique allows reducing extraction times to 20 minutes or less because of the high rate of penetration of supercritical CO_2 into biological matrices [30].

More recently, Ionic liquids (ILs) have been employed as extraction solvents in sample treatment, since their composition can be tailored, improving their, by its very nature, outstanding properties and behavior as extracting solvents. ILs are mainly made of organic cations and inorganic or organic anions, and the properties can be very different in relation to the length of the alkyl chain of the cation and to the type of anion. Liquid and solid-phase microextraction techniques can be improved by the employment of ILs instead of conventional organic solvents, reducing the negative effects on the environment and enhancing the throughput [31, 32, 33].

In this thesis, two different green sample treatment methods were explored for cleaning-up and extraction of biological matrices for veterinary, forensic, clinical and foodstuff applications: ACN-based protein precipitation (PPT), and supramolecular solvents (SUPRASs).

2. ACN- based treatment for protein precipitation in biological matrices (PPT)

Protein precipitation (PPT) is a traditional plasma treatment in fields such as clinical science and forensic science because it is a very cheap, easy, quick, and quite effective technique and, in addition, no specific equipment is required. Although PPT shows some lacks (i.e., low selectivity and high signal-suppression in LC-MS/MS), it is considered nowadays a golden standard in routinely sample treatment when the target analyte concentration is quite high. Because of these unique features, pharmaceutical companies regularly use PPT in their processes. Moreover, when target analytes are sensitive to light, heat and changes in pH (e.g.,

[29] L. Correia-Sá, S. Norberto, C. Delerue-Matos, C. Calhau, V.F. Domingues, J. Chromatogr. B, 1072 (2018) 9–16.
[30] A. Ríos, M. Zougagh, F. de Andrés, Bioanalysis, 2 (2010) 9–25.
[31] D. Han, K.H. Row, Molecules, 15 (2010) 2405–2426.
[32] H.F. Almeida, M.G. Freire, I.M. Marrucho, Green Chem., 19 (2017) 4651–4659.
[33] M. Yang, Y. Gu, X. Wu, X. Xi, X. Yang, W. Zhou, H. Zeng, H. Zhang, R. Lu, H. Gao, J. Li, Food Chem., 239 (2018) 797–805.

flavonoids or endogenous antioxidants) protein precipitation is the favorite procedure in sample treatment [34]. The process of "cleaning up and extraction" takes place is one easy step, where a water miscible organic solvent (e.g., acetonitrile, methanol or acetone) is mixed with an amount of sample causing the protein precipitation. Methanol is very used in protein precipitation because it is considered quite efficient, but precipitation achieved by acetonitrile is stronger than that for methanol and ethanol, that is, acetonitrile extracts contain lower concentrations of proteins than methanol extracts. Usually, precipitation is improved by a centrifugation step (Figure 4). The mechanism which causes precipitation is due to the interference of the organic solvent with protein structure altering intramolecular hydrophobic interactions and minimizing the hydration/solubility of proteins. Sometimes, the need to obtain a higher enrichment factor and reduce the amount of interferences leads to the evaporation of the extracted supernatant until dryness, and redissolution with a proper amount of solvent for further analysis [4, 12, 35, 36]. An important feature of PPT is that analytes are extracted whether they were previously bound to protein or not [34].

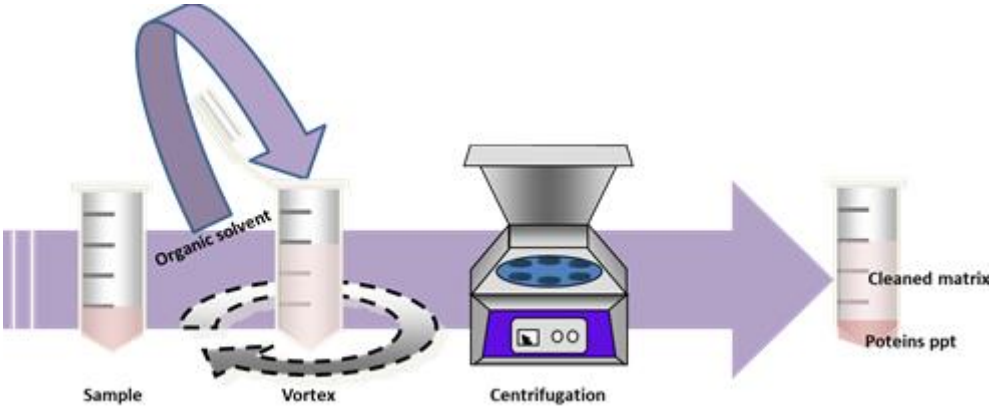

Figure 4. PPT procedure in plasma treatment.

To improve the throughput, aqueous precipitant solutions containing ammonium sulfate (saturated at room temperature), aluminum chloride (5%, w/v), m-phosphoric acid (5%, w/v), trichloroacetic acid (10%, w/v), zinc sulfate heptahydrate (10%, w/v) and 0.5 M sodium hydroxide, has also been employed instead of traditional organic solvents. Agent/ sample ratios are essential for obtaining a satisfactory precipitation (removal of proteins >98%). Acid/plasma ratios are usually in the range of 0.2–0.5:1.0 (v/v), while for organic solvents/plasma the range is 1.0–4.0:1.0 (v/v) (Figures 5, 6) [35].

[34] J.H. Oh, Y.J. Lee, Phytochem. Anal., 25 (2014) 314–330.
[35] L. Nováková, H. Vlčková, Anal. Chim. Acta, 656 (2009) 8–35.
[36] N.Y. Ashri, M. Abdel-Rehim, Bioanalysis, 3 (2011) 2003–2018.

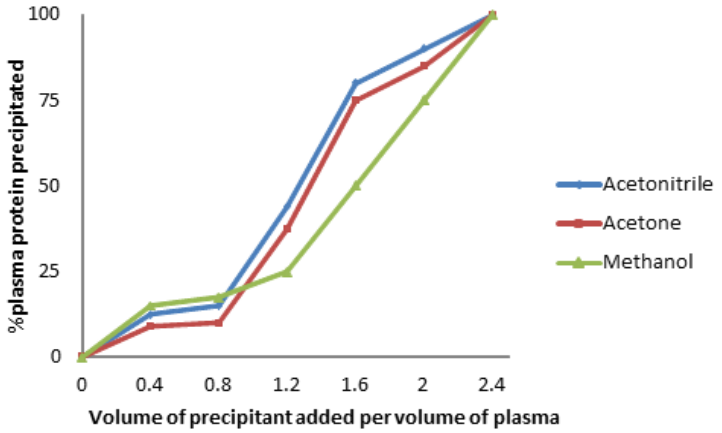

Figure 5. Graphical representation of the activity of organic solvents in protein precipitation.

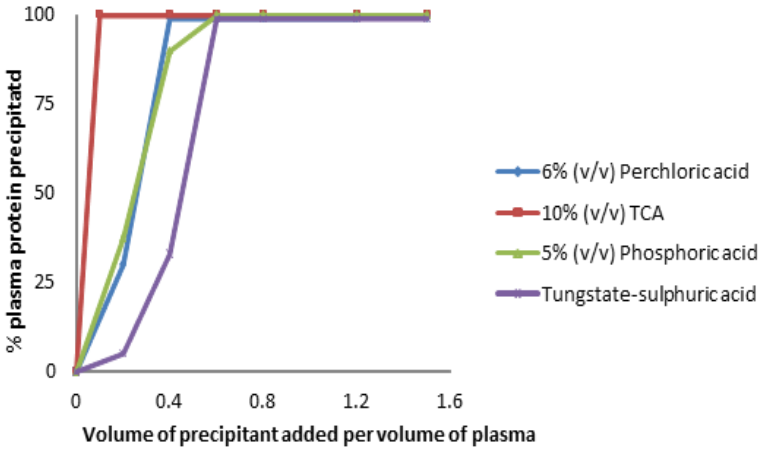

Figure 6. Graphical representation of the activity of acids in protein precipitation.

The automatization of PPT was achieved by J. Ma et al. in 2008 by means of a 96-well plate format followed by LC-MS/MS for application in pharmacokinetic and pharmacodynamic studies. In this work, on-deck plate shakers, centrifuges, plate sealers, and plate seal piercing stations were employed to totally avoid the handling work of the operator [37].

In this thesis a cheap, simply, rapid, and green ACN-based treatment for protein precipitation in plasma was employed for the detection of light and air sensitive compounds such β-carotene, retinol and α- tocopherol for clinical and veterinary applications.

[37] J. Ma, J. Shi, H. Le, R. Cho, J.C.J. Huang, S. Miao, B.K. Wong, J. Chromatogr. B, 862 (2008) 219–226.

3. Supramolecular solvents (SUPRAS)

Supramoleculecular solvents (SUPRAS) are nanostructured liquids generated from a spontaneous process of self-assembly and coacervation.

The process of coacervation, which allows SUPRAS formation, was described by the colloidal scientists Bungenberg de Jong and Kruyt in 1929 [38]. In analytical chemistry, it was firstly introduced as cloud point extraction (CP) by Watanabe and Tanaka [39].

Coacervation consists in a separation process where colloidal systems are separated in two liquid phases, where one of them is very rich in colloid, and the other one is an equilibrium solution. These systems have been studied in deep, and even new synthesis approaches are proposed everyday by researchers belonging to very different fields such colloid, polymer, physicochemical, and pharmaceutical sciences [40].

The main SUPRAS feature for analytical applications is that they can be tailor-made in regard to their physicochemical properties by changing the initial synthesis/self-assembly conditions (type of amphiphiles and environment). It is possible to synthetize an "a´ la carte" SUPRAS for a specific cleaning-up/extraction by considering the properties of the target analyte and the complexity of the matrix.

The amphiphilic character of SUPRAS is a fundamental point for sample treatment and analyte extraction. The nano-systems, which form the internal structure in SUPRAS, show two different polarity regions. In this way, several types of interaction coexist, which allow the solubilization / extraction of analytes showing a wide polarity range. Moreover, due to the high concentration of amphiphiles in SUPRAS (0.1-1 mg μL^{-1}), the number of binding sites for the target compound in also very high, rendering quantitative recoveries for a lot of different compounds [40, 41].

One of the main problems when quantifying and analyte in a biological matrix is its usually very low concentration, especially when compared with the interferences. SUPRAS are able to preconcentrate the target analyte up to 500 times the initial concentration (typically 100-500) and, additionally, to exclude interferences, since some of them can act as restricted

[38] H.G. Bungenberg de Jong, H.R. Kruyt, Kolloid Coacervation (Partial miscibility in colloid systems). In: Proceedings of the Section of Sciences, Kon. Akad. v. Wetenschappen, Amsterdam, 32 (1929) 849–856.
[39] H. Watanabe, H. Tanaka, Talanta, 25 (1978) 585–589.
[40] C. Caballo, M.D. Sicilia, S. Rubio, Supramolecular Solvents for Green Chemistry. In: F. Pena-Pereira, M. Tobiszewsky (eds) The Application of Green Solvents in Separation Processes. Elsevier, (2017).
[41] A. Ballesteros-Gómez, M.D. Sicilia, S. Rubio, Anal. Chim. Acta, 677 (2010) 108–130.

access materials (RAM). For all these reasons, SUPRAS are classified as a green sample treatment technique [40].

3.1. Synthesis of Supramolecular Solvents; Self-Assembly and Coacervation

The synthesis of SUPRAS is a very simply process carried out by a spontaneous process of self-assembly and coacervation. A colloidal solution of tri-dimensional aggregates is formed by an amphiphile solution above the critical aggregation concentration. In order to induce the coacervation process, an environmental change is required (e.g., a change of pH, temperature, addition of salt or a poor-solvent for the amphiphiles, etc.). Afterwards, aggregates grow in size, become bigger, and form the coacervate droplets, which associate with each other in conglomerates. In this way, creaming and phase separation are achieved and SUPRAS are formed. Coacervate droplets, which stay in equilibrium with the bulk solution at the critical aggregation concentration, continue to keep their initial integrity and characteristics in the SUPRAS (Figure 7) [41].

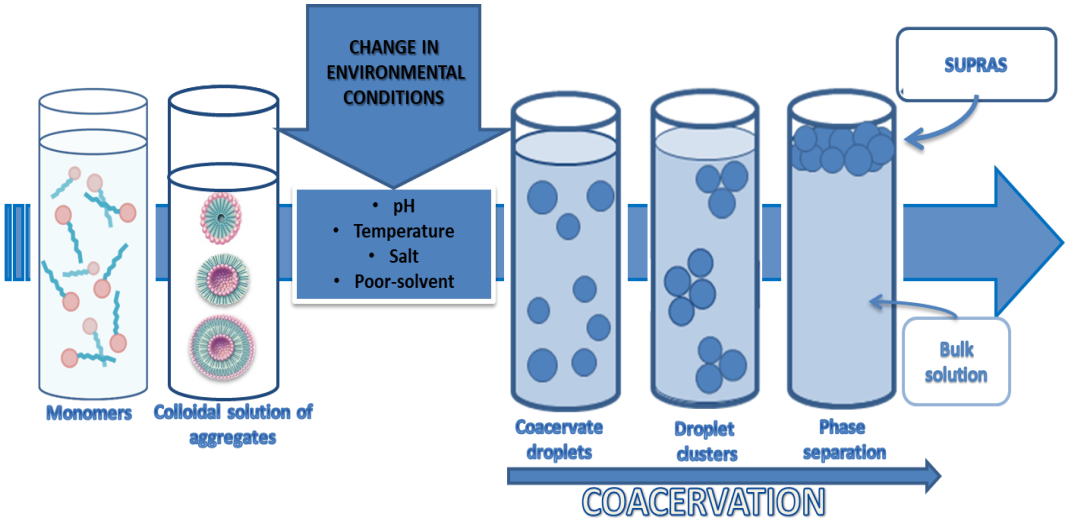

Figure 7. General syntheses of SUPRAS. A change in an environmental condition induces the coacervation process.

Self-assembly is defined as a spontaneous and totally reversible process, which ends with the formation of ordered structures. The setting of these structures depends on the features of the monomers and the environmental conditions involved in the self-assembly. Among others, the most important factors are the size of the polar head group and the length of the

lipophilic chain of the amphiphilic molecules. Thus, the morphology of the aggregates can be defined by calculating the packing factor (g) proposed by Israelachvili et al. in 1976 [42]:

$$g = \frac{V}{a_0 l_c}$$

where V is the volume of the hydrophobic chain, a_0 is the mean cross-sectional area of the head group and l_c is the length of the entirely extended chain. Figure 8 reports the different several morphologies that can be predicted by the packing factor [41].

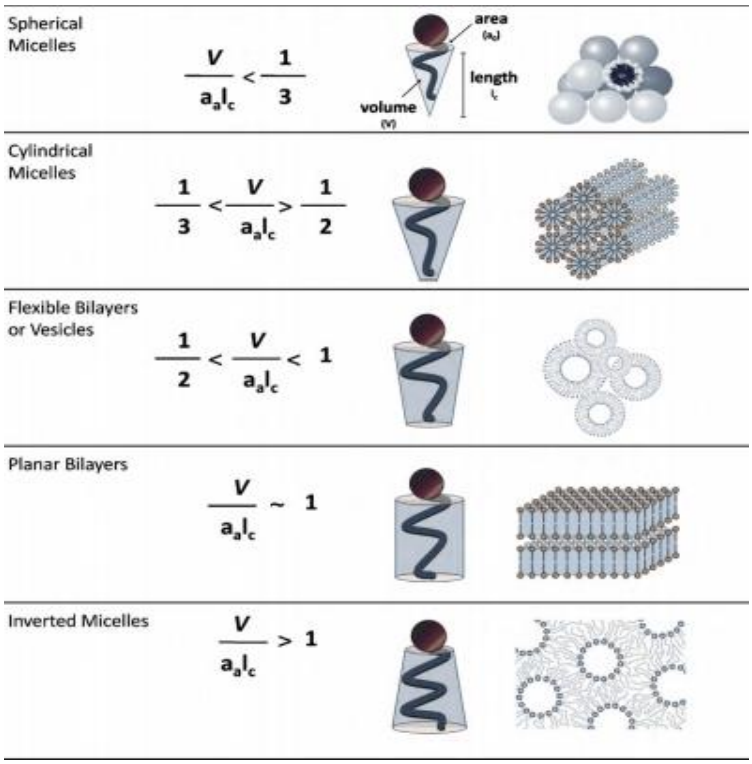

Figure 8. Several morphologies predicted by the packing factor of amphiphiles.

Attractive and repulsive forces have a fundamental role during the process. They arise from non-covalent and reversible interactions with energy values of 2-300 kJ/mol (e.g., coulomb, van der Waals, π-π and π-cation interactions, hydrogen bonding and dispersion) [43]. If the environment changes, the system adapts to the new conditions, being this the key why SUPRAS are tailor-made solvents [44]. The gateway of the process is aggregation and,

[42] J.N. Israelachvili, D.J. Mitchell, B.W. Ninham, J. Chem. Soc. Farad. T 2: Molecular and Chemical Physics, 72 (1976) 1525–1568.
[43] J.W. Steed, D.R. Turner, K.J. Wallace, Introduction. In: J.W. Steed, D.R. Turner, K.J. Wallace (eds) Core Concepts in Supramolecular Chemistry and Nanochemistry. John Wiley & Sons, (2007).
[44] J.M. Lehn, Eur. Rev., 17 (2009) 263–280.

typically, solvophobicity promotes SUPRAS formation, while the repulsion forces of the hydrophilic heads terminate it [45].

Micelles, vesicles and reverse micelles represent three possible morphologies for the ordered structures. The physicochemical properties of the amphiphiles, the environmental conditions and the nature of solvents drive for one among these three morphologies. Anyway, in aqueous colloidal solutions micelles and vesicles are preferentially formed, while in non-aqueous colloidal solutions reverse micelles are preferred (Figure 9) [41].

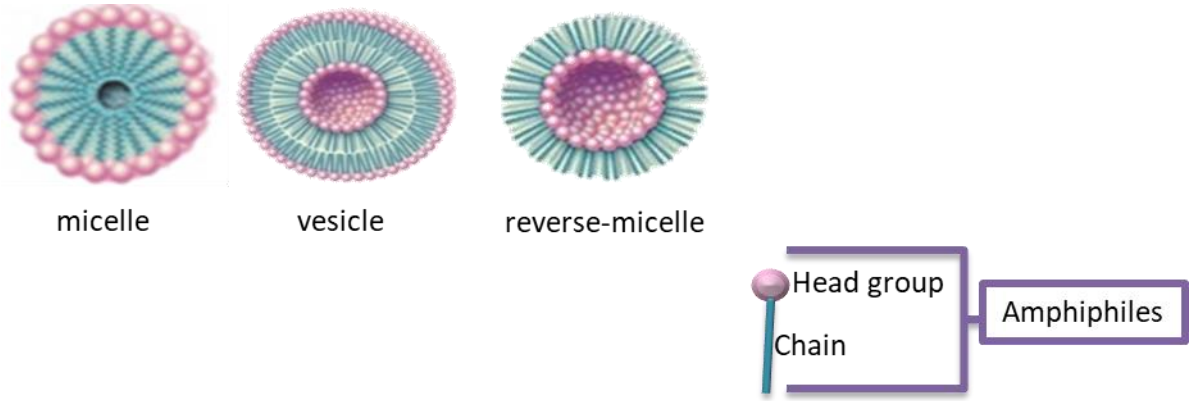

Figure 9. Different types of ordered structures in colloidal solution.

After these colloidal solutions are formed, aggregates must become bigger to overcome the repulsion forces which stop aggregation. To obtain coacervate droplets, an environmental change is necessary, being the approach different if the amphiphile is neutral or ionic. [40].

Coacervation is achieved in ionic systems by the addition of coacervating agents, or by changing the pH of the solution. In case of nonionic colloidal system, coacervation can be carried out by modifying the temperature of the solution or by the addition of a poor solvent for the amphiphilic molecules; these two procedures reduce solvation and promote the formation of coacervate droplets [40, 46].

When a new SUPRAS is synthetized or applied for the first time to a matrix, all these experimental conditions are evaluated by the construction of phase diagrams, where the amphiphile is reported as function of the coacervating agent.
Following, all the strategies for inducing coacervation are reported in deep.

[45] D.F. Evans, H. Wennerström, Solutes and Solvents, Self-Assembly of Amphiphiles. In: D.F. Evans, H. Wennerström (eds) The Colloidal Domain: where Physics, Chemistry, Biology, and Technology Meet. Wiley-VCH, (1999).
[46] J.A. Pelesko, Engineered Systems. In: J.A. Pelesko (eds) Self-Assembly: The Science of Things that Put Themselves Together, Chapman & Hall/CRC, (2007).

Temperature is a very helpful coacervating agent for nonionic, zwitterionic, and mixtures of nonionic and nonionic/ionic systems. The temperature which induces turbidity and aggregation in colloidal solutions is called cloud point (CP) [47, 48]. Phase diagrams obtained for Triton X-114, and for 3-(nonyldimethylammonium) propyl sulfate (C_9-$APSO_4$), both in aqueous solutions, are shown in Figure 10 [40].

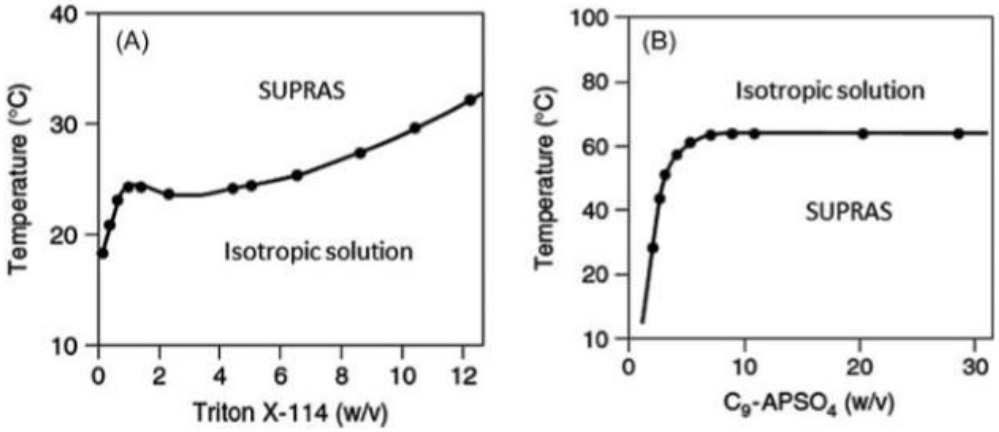

Figure 10. Phase diagrams of a nonionic system (A) and a zwitterionic system (B) in function of the temperature.

The principle which allows obtaining SUPRAS from nonionic surfactants (alkyl and alkylphenol ethoxylated) is desolvation of the area which surrounds the polar groups. In this way, the polar heads are reduced in size, and interactions among the neighbor micelles are facilitated. These interactions cause micellar growth and promote SUPRAS formation.

CP is inversely proportional to the hydrocarbon chain length and directly proportional to the number of oxyethylene groups of the surfactant [49, 50]. CP is also inversely proportional to the amount and the type of electrolyte (e.g., $PO_4^{-3} > SO_4^{-2} > Br^-$), which can be employed to enhance the process. CP is increased by the addition of nonpolar organic compounds which are solubilized in the micellar core, but it is negative affected by polar compounds, which are solubilized in the surface of the micelle [51, 52].

[47] B.M. Cordero, J.L. Pavón, C.G. Pinto, M.E. Laespada, Talanta, 40 (1993) 1703–1710.
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[52] M.J. Rosen, Micelle Formation by Surfactants. In: M.J. Rosen (eds) Surfactants and Interfacial Phenomena. John Wiley & Sons Ltd, (2004).

When a SUPRAS is made up of more than one non-ionic surfactant, it is possible to calculate the CP as the average of the CPs of each surfactant; however, when the mixture consists of nonionic/ionic surfactants, CPs values are higher than those for the individual solutions because of the repulsion forces of the ionic heads [53, 54].

As showed in Figure 10.B, SUPRAS formation regions for zwitterionic surfactants are below a critical value of temperature. In fact, the presence of polar groups does not influence the CP as in ionic surfactants because electrostatic interactions of zwitterionic compounds are negligible. [48].

Water is a very commonly used coacervating agent for water-insoluble nonionic surfactants. The procedure consists in solubilizing the amphiphile in a proper organic solvent and afterwards, adding water, which promotes coacervation. This technique has been employed for carboxylic acids-based and alkanols-based SUPRAS. Furthermore, because the amount of water in liquid biological matrices is very high, this approach allows synthetizing the SUPRAS *in situ* with excellent throughputs [12, 55].

There are several organic solvents that can be used for solubilizing the amphiphiles, e.g., dioxane, tetrahydrofuran (THF), acetone, acetonitrile, ethanol, methanol, propanol, ethylene glycol, dimethylformamide, etc. [55]. The SUPRAS formation region is proportional to the dielectric constant of the solvent and the length of the hydrophobic chain of the surfactant. An example of this behavior is reported in Figure 11, where the formation region of a decanoic acid-based SUPRAS is shown to be much smaller in ethanol (A) than in tetrahydrofuran (B). Water-induced SUPRAS are not affected by changes in temperature or by the presence of electrolytes. All their outstanding properties depend on the initial synthesis composition of the properly selected ternary mixture [40].

[53] T. Inoue, H.J. Ohmura, Colloid Interf. Sci., 258 (2003) 374–382.
[54] T. Gu, P.A. Galera-Gómez, Colloids Surf. A: Physicochem. Eng. Aspects, 104 (1995) 307–312.
[55] F.J. Ruiz, S. Rubio, D. Pérez-Bendito, Anal. Chem., 79 (2007) 7473–7484.

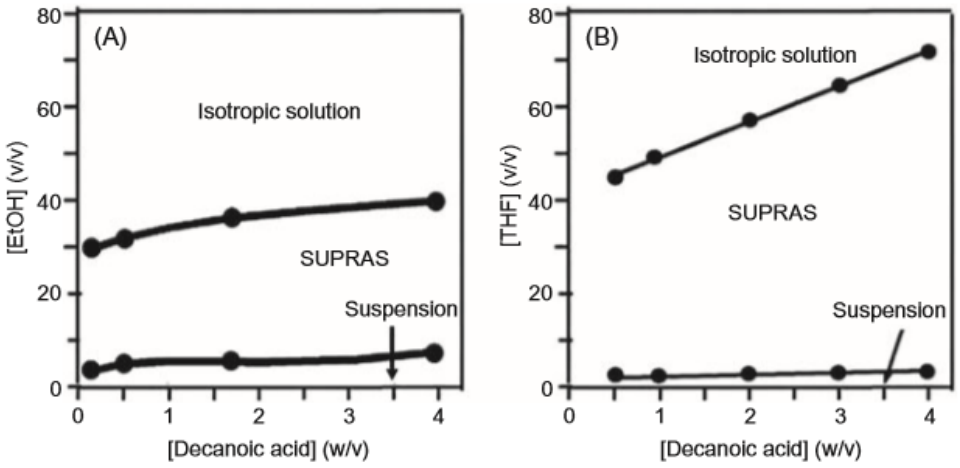

Figure 11. Phase diagrams of mixtures of decanoic acid/ethanol/water (A) and decanoic acid/tetrahydrofuran/water (B).

Acid-induced SUPRAS are based on processes where the change of pH of ionizable amphiphile in aqueous solution triggers the coacervation. The ternary mixture can be composed by alkyl sulfates, sulfonates, or sulfosuccinates mixed with HCl and water. Figure 12 shows the phase diagram of a SUPRAS made up of sodium dodecane sulfonate (SDoS) in acid aqueous solution [56, 57].

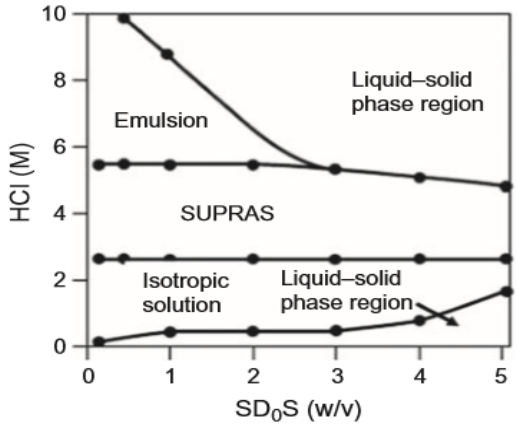

Figure 12. Phase diagram of a SUPRAS made up of sodium dodecane sulfonate (SDoS) in acid aqueous solution.

Colloidal solutions of ionic amphiphiles can be also coacervated by the addition of counterions. Sulfate and chloride are commonly used for this purpose. They trigger micellar

[56] I. Casero, D. Sicilia, S. Rubio, D. Pérez-Bendito, Anal. Chem., 71 (1999) 4519–4526.
[57] D. Sicilia, S. Rubio, D. Pérez-Bendito, Anal. Chim. Acta, 460 (2002) 13–22.

growth by reducing the repulsion forces of the polar heads (Figure 13). Vesicles of mixtures of carboxylic acids and carboxylates (1:1 w/w) in aqueous solutions coacervate efficiently by the addition of tetrabutylammonium counterions as shown in the phase diagram in Figure 13.B [58, 59, 60, 61].

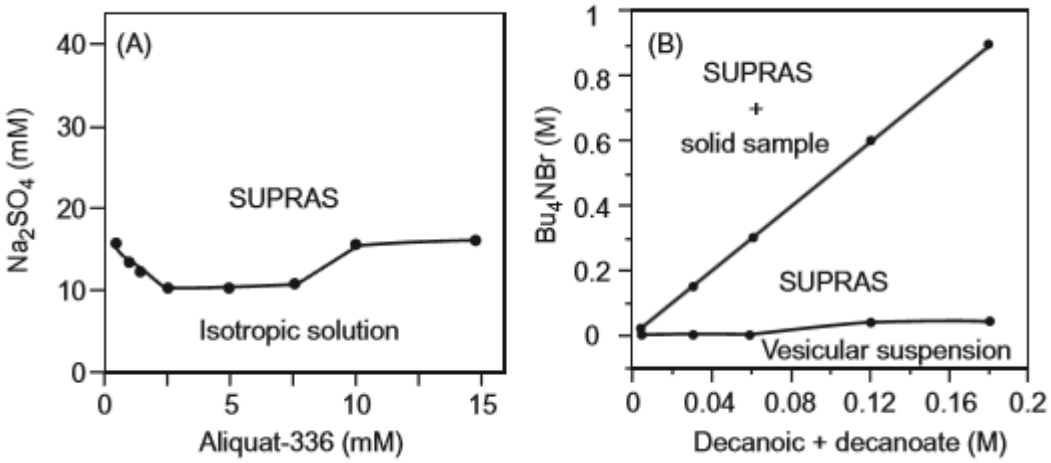

Figure 13. Phase diagram of tricaprylyl methylammonium chloride (Aliquat-336) in function of sodium sulfate (A), and phase diagram obtained from mixtures of decanoic acid: decanoate in the presence of tetrabutylammonium counterions (B).

3.2. Extracting properties of supramolecular solvents: solubilization and concentration factors

The main property of SUPRAS being able to solubilize several types of solutes is due to two factors: the mixed mode mechanism by means of which SUPRAS interacts, and the high concentration of amphiphiles in the solvent (0.1-1 mg μL^{-1}), which offers a large number of binding sites to the target analytes. Ionic interactions, hydrogen bonding, dipole-dipole interactions, and dispersion, are the typical interactions which are involved in SUPRAS extraction. The mixed mode mechanism is consequence of the coexistence of regions with different polarity in the ordered structure. Figure 14 shows an example of a hexagonal aggregate of reverse micelles from mixtures of hexanol/THF/water, where the polar-nonpolar regions are easily distinguished [12, 40].

[58] X. Jin, M. Zhu, E.D. Conte, Anal. Chem., 71 (1999) 514–517.
[59] B.K.W. Man, M.H.W. Lam, P.K.S. Lam, R.S.S. Wu, G. Shaw, Environ. Sci. Technol., 36 (2002) 3985–3990.
[60] B.L. Bales, R. Zana, Langmuir, 20 (2004) 1579–1581.
[61] F.J. Ruiz, S. Rubio, D. Pérez-Bendito, Anal. Chem., 78 (2006) 7229–7239.

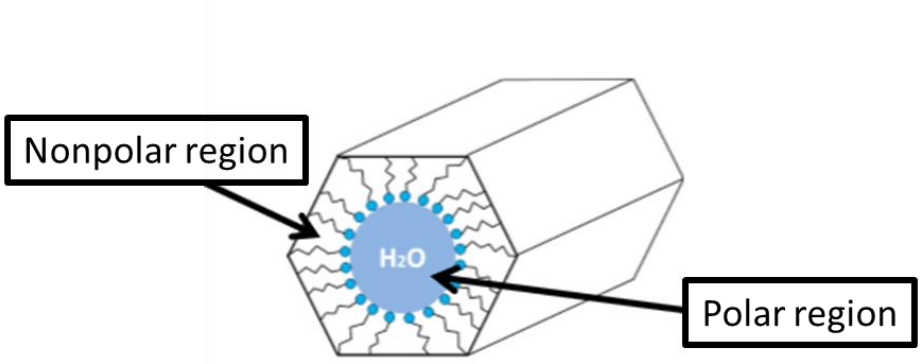

Figure 14 Polar/nonpolar regions of a Hexanol-based SUPRAS.

The efficiency of the extraction depends on octanol/water constants for nonpolar analytes and, in the case of amphiphilic analytes, in their ability to form co-aggregates [40]. Polar analytes are extracted trough ionic interactions, hydrogen bonding, π-cation interactions, and π-π interactions. Nevertheless, the extraction of very polar compounds is problematic. For example, alkyl sulfates and cetrimide, which are coacervated by the addition of HCl and NaCl, respectively, are not able to extract ionic solutes, because their nonionic behavior. Moreover, in the extraction of polar compounds, the length of the chain of the amphiphile and the CP are extremely important for obtaining quantitative yields [40].

SUPRAS are able to yield high enrichment factors (typically, 100-500). This property is a direct consequence of the volume of SUPRAS formed. In general, when the composition of SUPRAS is kept constant, the amount of solvent produced shows a linear dependency with the content of amphiphile in the bulk solution, for concentrations below 4% [62].

Two factors drive to final concentration of amphiphiles in SUPRAS: the molecular structure of the amphiphiles and the environmental sphere. Usually, concentration is inversely proportional to the length of the lipophilic chain (e.g., decanoic > dodecanoic > tetradecanoic in vesicles formed by alkylcarboxylic acid/tetrabutylammonium alkylcarboxylates). Moreover, when the surfactant is an ethoxylated molecule, also the number of oxyethylene groups influences the concentration, decreasing it [63].

[62] A. Ballesteros-Gómez, F.J. Ruiz, S. Rubio, D. Pérez-Bendito, Anal. Chim. Acta, 603 (2007) 51–59.
[63] P. Taechangam, J.F. Scamehorn, S. Osuwan, T. Rirksomboon, Colloids Surf. A: Physicochem. Eng. Aspects, 347 (2009) 200–209.

Differences between operating conditions and CPs in nonionic surfactants also induce more compact structures, which result in lower water contents and SUPRAS volumes [64, 65, 66].

Anyway, the final volume of SUPRAS formed can be calculated a priori by means of general equations, which can be obtained from experimental studies. Regretfully, they are specific for a particular SUPRAS [40].

The environmental sphere not only promotes SUPRAS formation, but it also influences in the final solvent composition. The addition of salts decreases the CP (e.g., $Na^+ > K^+ > Cs^+ > NH_4^+$, and $OH^- > F^- > Cl^- > Br^-$), but it increase salting-out (e.g., $PO_4^{3-} > SO_4^{2-} >$ monovalent anions) in nonionic micelles. Triton X-405 switches between CPs, from 115 °C to 45 °C or 85 °C by the addition of 0.4 M K_3PO_4 and 2.4 M KBr, respectively [52, 67]. In general, salts modify both CP and SUPRAS composition in a concentration-dependent way. In fact, SCN^-, I^-, Ag^+ and divalent cations increase the CP and therefore, the water content [52]. Furthermore, the addition of bases, acids, polymers, alcohols and organic compounds to the synthesis condition, can influence the CPs [68, 69].

3.3. SUPRAS extraction formats and compatibility with separation and detection techniques.

SUPRAS extraction procedure can be carried out by *in situ* synthesis or *ex situ* synthesis. In the first case, taking advantage of the high amount of water usually present in biological liquid samples, the process of self-assembly and coacervation for the formation of SUPRAS is directly obtained in matrix by the addition of the amphiphile solution (0.1-2%), and using proper environmental conditions. Stirring and centrifugation are subsequently employed (Figure 15). In this way, analytes are directly extracted and cleaned-up from a complex matrix by an easy single step. Sometimes, to further eliminate matrix interferences and/or the presence of water, an evaporation step is required [40, 41]. Ionic surfactants-, carboxylic acid- and alkanol-based SUPRAS are typically formed in the upper layer, and their separation is very simple [40].

[64] R. Carabias, E. Martínez, B. Rodríguez-Gonzalo, J.L. Moreno-Cordero, C. Pérez-Pavón, E. García-Pinto, E.F. Laespada, J. Chromatogr. A, 902 (2000) 251–265.
[65] D. Sicilia, S. Rubio, D. Pérez-Bendito, Anal. Chim. Acta, 460 (2002) 13–22.
[66] E.W. Crick, E.D. Conte, J. Chromatogr. A, 877 (2000) 87–93.
[67] H. Akbas, C. Batigöc, Fluid Phase Equilib., 279 (2009) 115–119.
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[69] T. Gu, P.A. Galera-Gómez, Colloids Surf. A.: Physicochem. Eng. Aspects, 147 (1999) 365–370.

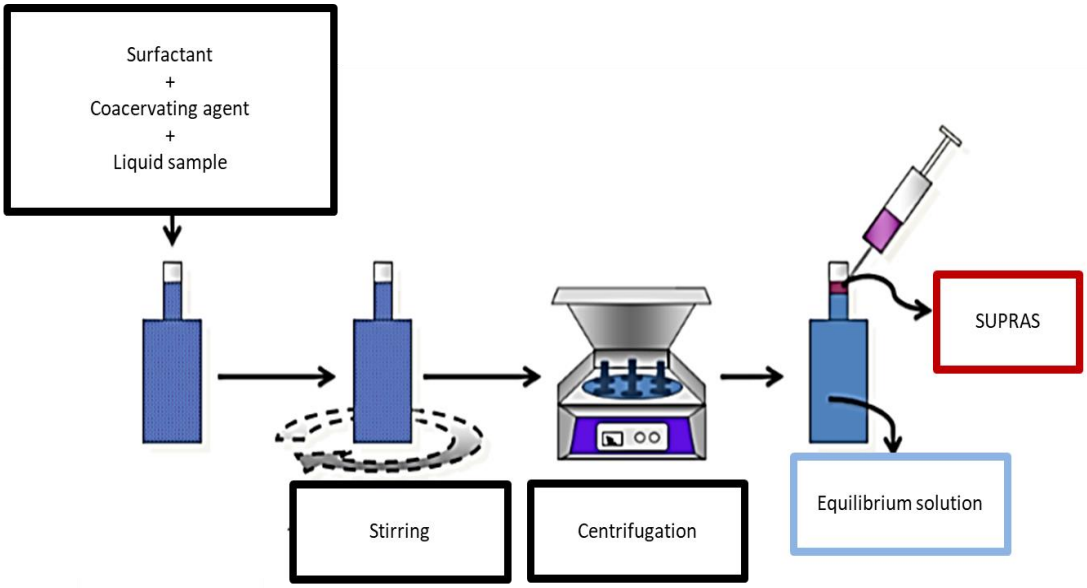

Figure 15. *In situ* SUPRAS synthesis procedure.

When the matrix is a solid (0.1-1 g), *in situ* or *ex situ* syntheses can be employed (Figure 16). In the *in situ* format, the extraction process is achieved in the same way as it has been explained above for liquid samples. Analytes are extracted as a result of an equilibrium among the three phases. Thus, nonpolar compounds, which are very soluble in the solvent and not in water, are efficiently extracted [40, 41]. The *ex situ* procedure was developed as a way to improve the throughput of the process, especially for polar compounds. The strategy consists in previous synthesis of the SUPRAS that, afterwards, is added to the solid sample. This format has allowed to obtain quantitative recoveries for herbicides in soils, and for polycyclic aromatic hydrocarbons (PAHs) in foodstuff [70, 71].

[70] F.J. López-Jiménez, A. Ballesteros-Gómez, S. Rubio, Food Chem., 143 (2014) 341–347.
[71] C. Caballo, M.D. Sicilia, S. Rubio, Talanta, 119 (2014) 46–52.

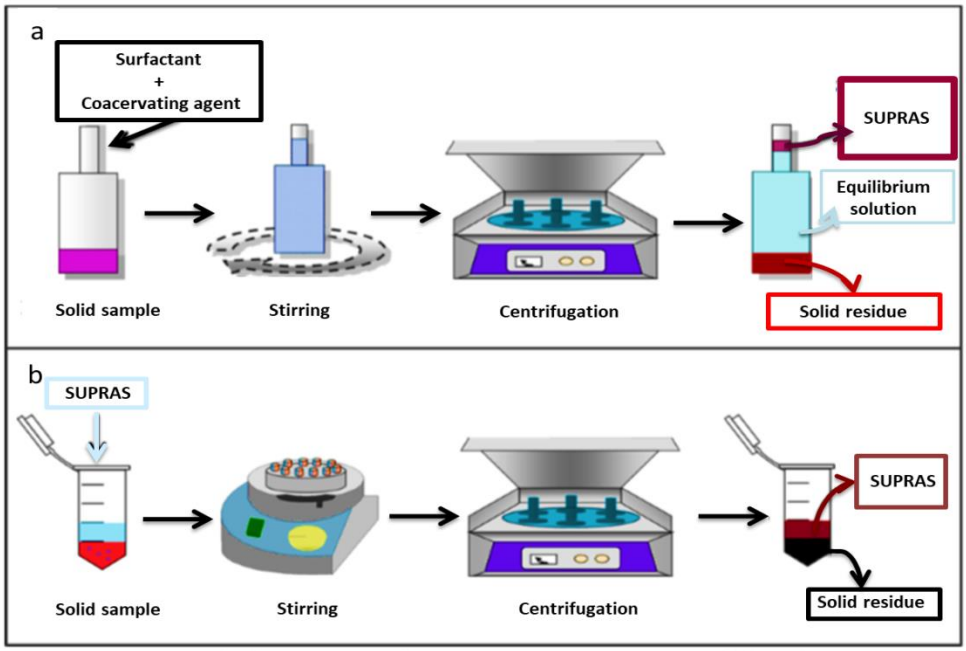

Figure 16. SUPRAS extraction for solid samples: *in situ* SUPRAS synthesis (a) and *ex situ* SUPRAS synthesis (b).

Another approach for SUPRAS extraction is single-drop microextraction (SDME). This format has been employed for the determination of chlorophenols in environmental waters. For this purpose, alkylcarboxylic acid-based SUPRAS were employed (Figure 17) [72].

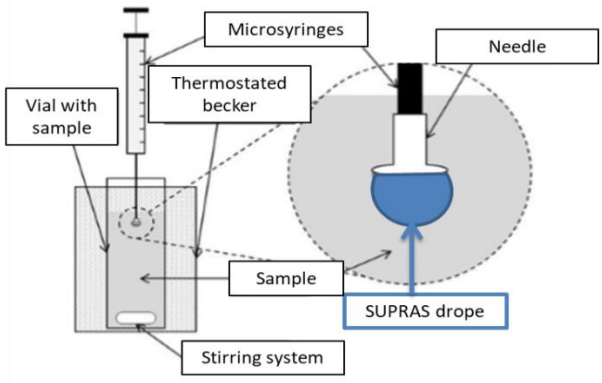

Figure 17. SUPRAS extraction as single-drop microextraction.

An on-line SUPRAS extraction/flow injection analysis (FIA) methodology has been proposed as an automatized procedure for the determination of PAHs by LC. The sample is mixed with surfactants and salts (for salting-out) and a collection column, made up of cotton

[72] F.J. López-Jiménez, S. Rubio, D. Pérez-Bendito, J. Chromatogr. A, 1195 (2008) 25–33.

and where SUPRAS is entrapped, is used. Subsequently, the elution step is achieved by an organic solvent. Lower enrichment factor than initially calculated theoretical values were obtained due to the dispersion of the analytes during elution [73].

During the years, the compatibility of SUPRAS with different separation and detection techniques has been demonstrated. Liquid chromatography followed by UV–vis, fluorescence and mass spectrometric systems has been mainly used. SUPRAS can be directly injected in LC, since aggregates disassemble in the mobile phase and do not affect the expected behavior of the analytes within the chromatographic system. When the mobile phase consists of an amount of water >40%, SUPRAS disassembly is slower and micelles can work as a pseudophase, which induces variations in the chromatographic behavior. To overcome this problem, SUPRAS can be diluted with a small quantity of organic solvent prior to their injection. The nature of the surfactant forming the SUPRAS is the key point for obtaining good results in separation and quantification. For instance, polyoxyethylene (n) tert-octylphenyl ethers (Triton X series) and polyoxyethylene (n) nonyl phenyl ethers (PONPE series), which are nonionic surfactants, induce signals in UV and fluorescence systems and, moreover, they can co-elute with polar or mid-polar analytes. These compounds can be easily extracted by SUPRAS which are formed by non-aromatic ionic or zwitterionic surfactants which overcome these difficulties.

For mass spectrometric systems, it is recommended to avoid the introduction of the SUPRAS into the detector, thus avoiding source contamination and SUPRAS related matrix effects [40].

Different strategies have been developed to coupled SUPRAS with gas chromatography. The main strategy consists in the removal of the solvent prior to the chromatographic injection. Triton X-114 is the surfactant usually employed in these methods and its removal is achieved by the use of single cation exchange, silica gel and Fluorisil columns or by the assistance of microwave or ultrasonic techniques for analyte back-extraction in a water-immiscible solvent [74, 75, 76, 77, 78, 79, 80]. A relatively new strategy to employ SUPRAS approach in gas

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chromatography, avoiding the separation step, is based on post-extraction derivatization of the surfactant before the injection into the system. N,O-bis(trimethylsilyl)trifluoroacetamide was proposed as derivatizing agent [81].

In spite of surfactants being usually employed in micellar electrokinetic chromatography (MEKC), the coupling of capillary electrophoresis (CE) and SUPRAS techniques has limited applications, even though supramolecular solvents are also well-matched with capillary electrochromatography (CEC) background electrolytes. The drawback of SUPRAS/CE methodologies is the clogging of the capillary. To avoid this issue, a post-extraction/dilution with an organic solvent is needed, but pseudo stationary phase formation keeps existing, which results in no reproducible migration times [82, 83]. In capillary zone electrophoresis (CZE), water can induce absorption of the surfactant onto the wall of the capillary. To overcome this problem, non-aqueous media can be employed (NACE) when combining SUPRAS-based extractions with CZE [84, 85].

SUPRAS extractions can be also coupled to MEKC. A dilution step of the SUPRAS extract with an organic solvent is needed for reducing the viscosity [86, 87, 88]. As for gas chromatography, a good strategy is to remove the amphiphile before injection in the CE instrument [89, 90, 91].

3.4. SUPRAS applications in analytical chemistry

During the last decade, supramolecular solvents have been employed for the extraction of organic compounds and metals in environmental, food and biological matrices.

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3.4.1. Aqueous micelle-based SUPRAS

Taking into consideration the nature of the surfactant which forms the structure, SUPRAS made up by aqueous micelles can be subdivided in three subtypes:

- Non-ionic micelles-based SUPRAS
- Ionic micelles-based SUPRAS
- Mixed micelles-based SUPRAS

SUPRAS formed by nonionic surfactants and obtained by an increase of temperature have been the most employed SUPRAS in sample extraction over the last decade. This wide use seems to be related to the limited efforts to develop new strategies on SUPRAS methodology. The most used surfactants for these applications have been Triton X-114, Triton X-100 and Genapol X-080, at working concentrations around 1%, or around 3-10% for the extraction of bioactive compounds in biological matrices. The pre-concentration factors which can be reached with this type of SUPRASs are quite low (1-10), although it is possible to increase them by changing the environmental conditions (e.g., addition of salts). Thus, pre-concentration factors up to 73-152 have been achieved for the extraction of hormones with Triton X-114 (0.25%) with the addition of a salt (0.4M Na_2SO_4) at 45 ºC [92].

Among SUPRAS formed by ionic micelles, acid medium-based ones have proved their suitability for the extraction of cations, even under extreme experimental conditions (e.g. 3–4 M HCl or 4 g NaCl) in solid samples such as soil, sludge and sediments [93]. Moreover, SUPRAS obtained by the use of acid media have shown high pre-concentration factors in aqueous matrices at low surfactant concentrations (e.g., 140 for 0.1% of dodecane sulphonic acid) [94]. An interesting aspect for LC-MS/MS applications is that anionic surfactants can be purchased as single homologues that show a single peak at low retention times in LC [40]. SUPRASs formed by cationic surfactants has been used with great results for the determination of chlorophenols in environmental water [95].

Mixed micelles-based SUPRASs are formed by nonionic and ionic surfactants, and they have been successfully employed for the extraction of charged analytes. Usually, the mixture is composed by Triton X-114 and Cetyl trimethylammonium bromide (CTAB) and Sodium

[92] L. Wang, Y.Q. Cai, B. He, C.G. Yuan, D.Z. Shen, J. Shao, G.B. Jiang, Talanta, 70 (2006) 47–51.
[93] M. Cantero, S. Rubio, D. Pérez-Bendito , J. Chromatogr. A, 1046 (2004) 147–53.
[94] D. Sicilia, S. Rubio, D. Pérez-Bendito, N. Maniasso, E.A.G. Zagatto, Anal. Chim. Acta, 392 (1999) 29–38.
[95] X. Jin, M. Zhu, E.D. Conte, Anal. Chem., 71 (1999) 514–517.

dodecyl sulfate (SDS) for extracting anionic and cationic analytes (e.g., pesticides, dyes, humic and fulvic acids), respectively [96, 97, 98]. Mixed micelles-based SUPRAS formed by mixtures of two non-ionic amphiphiles have been used for the extraction of PAHs. Despite the high working temperature (78 °C), authors claimed some advantages when compared to single surfactant and/or ionic-nonionic mixtures. To name a few: higher surface activity, co-stabilizing and co-sensitizing features, and relatively better selectivity [99, 100].

3.4.2. Vesicle-based SUPRAS

SUPRASs formed by vesicles of carboxylic acid are very rich in amphiphiles (1 g mL^{-1}), which results in better performances and higher pre-concentration factors (e.g. >700). They have showed great capability for the extraction of pesticides in food samples and endocrine disruptors, pesticides and phenols in environmental water samples [101, 102, 103, 104]. Their excellent performance is due to the high number of interactions (i.e., ionic, hydrogen bonding, π –cation and hydrophobic) with which analytes are solubilized. Moreover, the presence of strong cohesive forces among their molecules allows their application in drop microextractions formats [40].

3.4.3. SUPRAS with restricted access properties (RAM-SUPRAS)

A common feature shown by all supramolecular solvents is that the process of self-assembly and coacervation is spontaneous and reversible. Environmental changes trigger the process by stopping the repulsion forces among the head groups, so aggregates become bigger and coacervate is formed. The size of aggregates depends on the balance between repulsion forces among the polar heads and attraction forces among lipophilic chains. Thus, the size of aggregates can be tailored with the consequence that structures with selected properties can be properly designed for specific applications (functional SUPRAS). In this context, SUPRAS with restricted access properties (RAM-SUPRASs) were first proposed by A.

[96] A.R. Zarei, Anal. Biochem., 369 (2007) 161–167.
[97] N. Pourreza, M. Zareian, J. Hazard. Mater., 165 (2009) 1124–1127.
[98] J.C.A. de Wuilloud, R.G. Wuilloud, B.B.M. Sadi, J.A. Caruso, Analyst, 128 (2003) 453–458.
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[100] B. Delgado, V. Pino, J.H. Ayala, V. González, A.M. Afonso, Analyst, 130 (2005) 571–577.
[101] S. García-Fonseca, A. Ballesteros-Gómez, S. Rubio, D. Pérez-Bendito, Anal. Chim. Acta, 617 (2008) 3–10.
[102] A. Moral, M.D. Sicilia, S. Rubio, Anal. Chim. Acta, 650 (2009) 207–213.
[103] F.J. Ruiz, S.Rubio, J. Chromatogr. A, 1163 (2007) 269–276.
[104] A. Moral, M.D. Sicilia, S. Rubio, J. Chromatogr. A, 1216 (2009) 3740–3745.

Ballesteros-Gómez et al. [105]. These solvents are made up of alkanols (C6-C14) and THF, and coacervation is induced by the addition of water. They are formed by inverted hexagonal aggregates, where the hydrophilic heads surround the aqueous cavities, while the lipophilic chains are solved in THF (Figure 18).

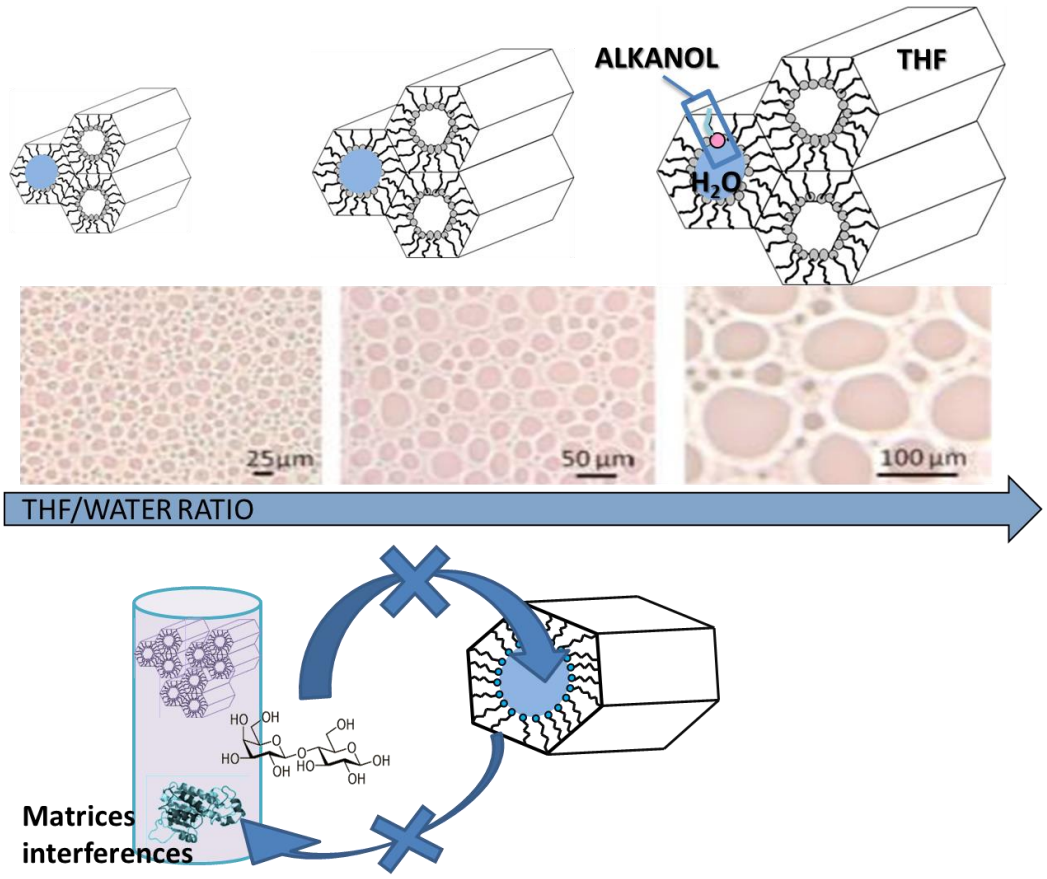

Figure 18 Schematic representation of structure, environmental responsive, and restricted access properties of alkanol-based SUPRAS.

The size of the aqueous cavity depends on the THF/water ratio, and it is a key point for the restricted access material activity, and for the extraction of polar compounds [105]. RAM-SUPRAS are able to extract solutes with low molecular weight and exclude, trough chemical (THF and alkanol protein precipitation) and physical (size exclusion of polysaccharides) mechanisms, big molecules such as the usual interferences in complex biological matrices (e.g. proteins, carbohydrates, humic acids etc.) [12, 106, 107]. In order to eliminate phospholipids, other common interference in biological matrices, an additional step of

[105] A. Ballesteros-Gomez, S. Rubio, Anal. Chem., 84 (2012) 342–349.
[106] S. Garcia-Fonseca, A. Ballesteros-Gomez, S. Rubio, Anal. Chim. Acta, 935 (2016) 129–135.
[107] F.J. Lopez-Jimenez, A. Ballesteros-Gomez, S. Rubio, Food Chem., 143 (2014) 341–347.

evaporation of the extracts prior to the injection in LC-MS/MS system may be applied (RAM-VOL-SUPRAS). Solutes are extracted by a synergism of interactions with a mix mode mechanism which consists both in polar and hydrogen bond interactions with alcohol heads, and dispersion interactions with the lipophilic chains.

Alkyl carboxylic acids (C8-C16 and C18) have been also employed for the synthesis of RAM-SUPRAS in a wide range of solvents (e.g., ethylene glycol, methanol, ethanol, 1-propanol, tetrahydrofuran, N,N-dimethylformamide, acetonitrile, acetone and dioxane), and with water as coacervating agent. Since carboxylic acids must be protonated to coacervate as SUPRAS (pKa R-COOH= 4.8 ± 0.2), acidic conditions are required for the synthesis, being the extraction carried out at pH<4 [103]. Acid- based SUPRASs with restricted access properties have been successfully proposed for the extraction of a wide range of compounds in food, environmental and clinical applications [12].

Part A

Determination of air and light sensitive endogenous antioxidants in serum by protein precipitation and high-performance liquid chromatography

Chapter 1

Blood serum retinol levels in Asinara white donkeys reflect albinism-induced metabolic adaptation to photoperiod at Mediterranean latitudes

Blood serum retinol levels in Asinara white donkeys reflect albinism-induced metabolic adaptation to photoperiod at Mediterranean latitudes

M.G. Cappai, M.G.A. Lunesu, Francesca Accioni, M. Liscia, M. Pusceddu, L. Burrai, M. Nieddu, C. Dimauro, Gianpiero Boatto, W. Pinna
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ABSTRACT

Previous works on albinism form of Asinara white donkeys (Equus asinus) identified the mutation leading to the peculiar phenotype spread to all specimens of the breed. Inbreeding naturally occurred under geographic isolation, on Asinara Island, in the Mediterranean Sea. Albino individuals can be more susceptible to develop health problems when exposed to natural sun radiation. Alternative metabolic pathways involved in photoprotection were explored in this trial. Nutrition-related metabolites are believed to contribute to the conservation of Asinara donkeys, in which melanin, guaranteeing photoprotection, is lacking. Biochemical profiles with particular focus on blood serum β-carotene and retinol levels were monitored. Identical natural grazing conditions for both Asinara (albino) and Sardo (pigmented) donkey breeds were assured on same natural pastures throughout the experimental period. A comparative metabolic screening, with emphasis on circulating retinol and nutrient-related metabolites between the two breeds, was carried out over one year. Potential intra- and interspecimen fluctuations of metabolites involved in photoprotection were monitored, both during negative and positive photoperiods. Differences ($p = .064$) between blood serum concentrations of retinol from Asinara versus Sardo breed donkeys (0.630 vs. 0.490 μg/ml, respectively) were found. Retinol levels of blood serum turned out to be similar in the two groups (0.523 vs. 0.493 μg/ml, respectively, $p = .051$) during the negative photoperiod, but markedly differed during the positive one (0.738 vs. 0.486, respectively, $p = .016$). Blood serum β-carotene levels displayed to be constantly around the limit of sensitivity in all animals of both breeds. Variations in blood serum concentrations of retinol in Asinara white donkeys can reflect the need to cope with seasonal exposure to daylight at Mediterranean latitudes, as an alternative to the lack of melanin. These results may suggest that a pulsed mobilization of retinol from body stores occurs to increase circulating levels during positive photoperiod.

KEYWORDS melanin, skin damage, sun radiation, vitamin A, β-carotene

1. INTRODUCTION

A worldwide acknowledged unique breed of feral albino donkeys originated in Sardinia, one of the major islands of the Mediterranean Sea. The Asinara white donkeys (Equus asinus, Linnaeus, 1758, var. albino) owe their name to the Asinara Island (N 41°4' 0.012", E 8°16' 0.012", Sardinia, Italy), established as National Park of the Autonomous Region of Sardinia, since 1998 (Official Gazette of Italian Republic, 1997) (Figure 1). The peculiar phenotype of Asinara white donkeys is characterized by a lifelong hypopigmentation of skin, hair, and eyes (Figure 2). The coat color has been definitely assessed [1], and the relative mutation has been recently elucidated [2].

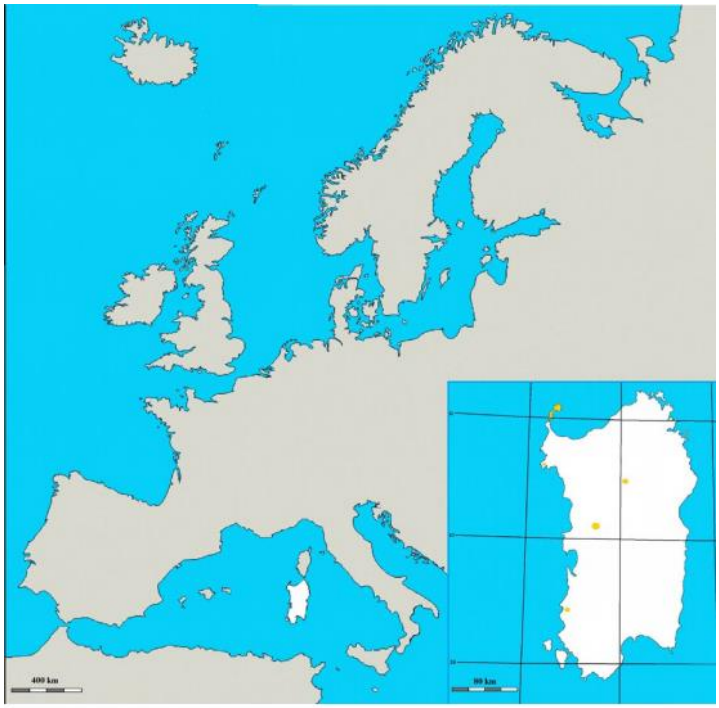

Figure 1 Map of Europe and magnification of Sardinia Isle (white), one of the largest islands of the Mediterranean Sea. In the frame on the right, the Asinara Island is colored in yellow and yellow spots on Sardinia highlight the presence of Asinara white donkeys in other regional parks. The National Registry of Local Minor Equine Breeds accounts 94 Asinara white donkeys living in Sardinia in 2015.

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Figure 2 Group of donkeys in the natural park in Is Arenas. Asinara white donkey (bottom left) and Sardo donkeys (bottom right and in the background) stepping toward bushes of Mediterranean vegetation. Picture taken during positive photoperiod

All specimens of Asinara breed display to possess an oculocutaneous form of albinism, classified as type 1 (OCA1). In OCA1 albinism, the phenotype can be due to the impaired conversion of L-tyrosine to L-3,4-dioxyphenylalanine (L-DOPA), in the early steps of the melanogenic process. The enzymatic inactivity [1] of tyrosinase (TYR) is now known to be due to the genetic mutation [2] of tyrosinase gene (Tyr). The consistency of Asinara white donkeys living on the Asinara Island is currently estimated to account 140 individuals. A lesser number of donkeys is distributed in other national parks of the Autonomous Region of Sardinia territory and, residually, in the rest of Italy (specimens: 294; conservation status: critical; records of the National Registry of Local Minor Equine Breeds, Association of Italian Breeders, 2016). Previous phylogenetic analyses [3, 4] suggested that the fixation of the mutation for albinism has been favored by geographical isolation. The autochthon origin of Asinara breed was supported by the molecular analyses carried out by Pinna et al. (1998)[4] who reported that albino donkeys seem to have branched out of the autochthon pigmented Sardo donkey, later studied also by Cosseddu et al. (2001) [3]. The first description of the Asinara white donkeys dates back to 19th century. This discovery can support the theory about the role exerted by geographic isolation and inbreeding phenomena. Inbreeding can naturally take place when genetically related individuals mate

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and offspring carry high levels of consanguinity. This may result in homozygosis for recessive alleles which are fixed in the next generations. The albinism of Asinara white donkeys has been recently identified to be due to a missense mutation in a highly conserved aminoacid position (G/G or D/D genotype), diverse from the pigmented phenotype (grey) of the Sardo donkey (C/C or C/G genotype) [2]. Inbreeding was also associated with isolated forms of albinism in other animal species [5, 6]. In individuals with different types of albino forms, the risk to develop skin cancer is reported to be higher than observed in pigmented individuals. In addition, this datum varies according to the type of albinism [5, 7, 8]. Other disorders are referred to ophtalmological problems like nystagmus or epiphora following direct sun exposure. Asinara donkeys show pink or light blue iris and unpigmented ocular fundus [1, 4]. As melanin is a skin, eye (retina and iris), and hair pigment with photoprotection properties against UV radiation, albino individuals may be naturally prone to photosensitivity and related complications. A high prevalence of secondary skin or eye disorders is reported to be linked to the form of albinism [9, 10, 11, 12]. Despite Asinara donkeys display OCA1 phenotype [1], the prevalence of skin or eye diseases is not reported to be higher than that detectable in other pigmented feral donkeys living in the same environment, except for photodermatitis at the top of the ears [1] and lower resistance to Myiasis cutanea [13]. Against this background, Asinara white donkeys' adaptation to the natural Mediterranean environment poses the question on how OCA1 does not seem to induce secondary diseases incompatible with life and responsible for low survival rates in nature, like skin cancer for instance, never reported in the literature to the best our knowledge. It appeared highly stimulating to understand how these albino animals successfully adapted to the Mediterranean climate and coped with environment and natural feeding stuffs. It was therefore hypothesized that endogenous factors, other than melanin, may play a decisive role in the natural metabolic adaptation of Asinara white donkeys. In the

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present work, we investigated whether variations in circulating retinol, as a biological measure to overcome the lack of pigment in skin, eye, and hair of Asinara white donkeys could be detected. Carotenoids consumed with the natural diet seem to be linked to circulating levels and distribution of metabolically active derivates in tissues of fish, birds, and mammals, and retinol may be one of these. In general, carotenoids may serve different purposes in the animal body, with different evolutionary meanings [14]. Dietary carotenoids display to possess diverse chemical structures. To date, over 600 known carotenoids compose this heterogeneous group of pigments, of which about 50 are useful substances for plants and animals. Carotenoids can be synthesized by plants, algae, and fungi, but mammals are incapable of de novo synthesis [15]. The diverse chemistry can be used to explain how different carotenoids enter diverse biochemical pathways. As a consequence, different physiological activities can lead to diverse biological effects of carotenoids after ingestion [15]. Due to their widespread presence in nature, carotenoids were the first phytochemicals studied for their ubiquitous functional roles [16]. Carotenoids can be classified into carotenes (hydrocarbons, likewise β-carotene and lycopene) and their oxidation products, known as xanthophylls. With regard to xanthophylls, they are known to contribute to the plumage of birds and coloration of fish. In particular, seasonal variations (nonmolt and molt, for instance) were observed in finches differently supplemented with dietary carotenoids [14]. To such an extent, the involvement of carotenoids in different aspects of life appears to be a common trait both for animals and plants. Vegetables and fruits are major sources of carotenoids for terrestrial mammals; however, other carotenoids (astaxanthin, for istance) can be synthesized by krill [17], thus being naturally available in the diet of marine animals and aquatic birds, like flamingos [18]. Natural pigments in the plant kingdom are associated with photosynthetic processes, as well as to dissipate energy excesses under light stress. The latter condition is mediated by a particular group of carotenoids [19, 20, 21]. Carotenes from plants, in particular β-carotene, display to possess pro-vitaminic properties for mammals. Vitamin A, or retinol, is synthesized starting from β-carotene in the small intestine and liver of animals. Vitamin A is known to take part, among other biological activities, in the visual

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function following light exposure. Vitamin A behaves as a strong antioxidant, capable to preserve cell membrane integrity. The role of retinol and β-carotene was explored by Wolf et al. (2000) in white recessive canaries, but no albino forms were associated in these birds. Thus, a comparative trial between Asinara white and pigmented Sardo donkeys, kept under same natural conditions and equally exposed to different intensities and duration of natural daylight, was carried out, with the attempt to clarify whether retinol might take part in photoprotection of albino specimens. Thus, this investigation was carried out over one year, to explore the metabolic adaptation to Mediterranean climate of albino specimens during different photoperiods.

2. MATERIALS AND METHODS

2.1 Animals and care

The investigation involved 11 stallions and 12 jennies, of which six of Asinara white and 17 of Sardo breed. All animals were individually recorded in the Official Register of the white donkey of Asinara (Ministerial Decree 27/7/1990) and of Sardo breed, respectively. The proportion of specimens from each breed enrolled in the trial is representative of the consistency of Asinara and Sardo donkeys, namely hundreds and thousands, respectively. All the experimental procedures presented in this study comply with recommendations of European Union directive 86/609/EEC and Italian law 116/92 concerning animal care.

2.2 Animals and farming conditions

All donkeys enrolled in the trial live altogether in the natural reserve of Is Arenas, in the south-western coast of Sardinia Island (N 41°4'0.012", E 8°16'0.012"). Individual blood sampling was carried for determining overall metabolic profile, with particular regard to β-carotene and retinol concentration, in two different periods (negative vs. positive photoperiod) throughout the year. Sampling periods were characterized by positive (peak in June, after three months of increasing light hours per day) and negative (month of October, after three months of decreasing light hours per day) photoperiod of the boreal hemisphere. All animals were individually and electronically identified by injectable transponders according to the EC Regulation 504/2008. Each donkey had free access to same natural pastures in both seasons. At each sampling, donkeys underwent the nutritional assessment [22] to estimate the nutritional state. Moreover, the comparative approach for the nutritional assessment was used to estimate any potential nutritional deficiency clinically manifest in

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both breeds. The trial lasted 12 months, which was considered a reliable period of time for avoiding biases due to bioaccumulation in the body of fat soluble dietary compounds from earlier seasons.

2.3 Blood sampling and laboratory analysis

Each donkey from both breeds was sampled for whole blood through the puncture of the jugular vein, to screen complete biochemical profile at start (October 2013) and end of the trial (October 2014), during negative photoperiod. Blood samples were also collected from same animals in the month of June 2014 (positive photoperiod), between the two negative photoperiods. For this purpose, all animals were gathered together in a paddock with mobile fences and moved into a corridor leading to a horse stock. Individual blood samples were cooled down and stored in tubes of polystyrene cases in the upright position in a cooling bag, to assure adequate temperature during the transfer of samples to the laboratory. All samples were labeled with the donkey name, electronic individual code (EIC), and date of sampling. All laboratory procedures on whole blood were started within six hours after collection. In field and laboratory protocols for the collection, storage and analyses of blood samples were carried out in the dark, in order to avoid photo-degradation of β-carotene and retinol. Individual serum was screened for complete biochemical profile. Prior to chemical analysis of blood serum, individual blood samples were centrifuged at 1500 × g for 10 min. An aliquot of individual serum was stored in a sterile vial (2 ml) and frozen at −20°C, until further analyses. All the samples were analyzed within one week, through an automatic biochemical analyzer (Mindray BS-200, Alcyon, Italy) for the determination of serum concentration of ubiquitous intermediate metabolites, enzymes, nutrients, macro- and micro-minerals. For the determination of β-carotene and retinol, high-pressure liquid chromatography coupled with an ultraviolet detector (HPLC-UV) was carried out. All standards and solvents were purchased from Sigma Aldrich (Milan, Italy). Stock solution (1 mg/ml) of β-carotene and retinol were prepared in methanol and chloroform/methanol (50/50), respectively. For the calibration curve, standard stock solutions were diluted with methanol and kept frozen at −20°C, protected from light. Serum levels of β-carotene and retinol were simultaneously measured at 325 and 450 nm, respectively. Chromatographic separation was carried out on a Waters Symmetry C18 column (4.6 × 150 mm, particle size 5 μm, Waters, Milford, Massachusetts). The injection volume was 20 μl. The mobile phases used were acetonitrile/methanol/Milli-Q water (64.5/33/2.5) at 1 ml/min for retinol, and 100% methanol at 2.8 ml/ min for β-carotene. Data were acquired and processed by Breeze

Software (Waters, Milford, Massachusetts). The limits of sensitivity for β-carotene and retinol were 50 and 100 ng/ml, respectively. Samples were prepared as follows: 0.3 ml of serum was vortexed with 0.6 ml of acetonitrile and centrifuged at 3500 × g at 4°C for 10 min. The supernatant was dried under a stream of nitrogen, and the residue was reconstituted in 0.15 ml of mobile phase [23, 24, 25, 26, 27].

2.4 Analysis of data and statistical methods

Data obtained on each sampling were analyzed using the following linear model:

$$Y_{i,j} = \mu + D_i + G_j + D_i \times G_j + e_{i,j}$$

where Y is the dependent variable (β-carotene and retinol concentration in blood serum), μ is the overall mean, D is the fixed effect of the sampling time (two levels: negative and positive photoperiod), G is the fixed effect of the coat color (two levels: pigmented vs. albino), D*G is the interaction factor, and e is the random residual. All data were analyzed using SAS 9.2 (SAS Inst. Inc. Cary, NC). The statistic significance was set for p-value < .05) were calculated for circulating Zn and total protein with retinol concentrations in blood serum. In both breeds, correlations were statistically analyzed because Zn and total protein were considered as nutrients related to intestinal absorption and conversion yields of β-carotene.

3. RESULTS

All animals involved in the trial appeared healthy. No signs of nutritional deficiency could be pointed out in both breeds. This finding was also supported by the optimal body condition score (BCS, based on a five-point scale, 1 = emaciation to 5 = obesity) recorded both in Asinara and Sardo donkeys (3.25 ± 0.15 vs. 3.50 ± 0.10, respectively). Biochemical profiles did not highlight significant differences between breeds (Table 1), as to parameters screened in this trial, except for retinol (Table 2). In fact, levels of retinol turned out to be significantly higher (+40.6% on average) in Asinara donkeys than those detected in blood serum of Sardo ones, during the positive photoperiod, whereas retinol concentrations (μg/ml) in blood serum appeared similar during negative photoperiods, in both the groups. The interaction between coat colour and photoperiod resulted to the limit of statistic significance (p = .051),

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as retinol blood serum concentrations in Asinara white donkeys set back to levels similar to those determined in Sardo donkeys during negative photoperiod. β-Carotene levels constantly resulted around the limit of sensitivity in all animals (50 ng/ml). Correlations between retinol and circulating zinc and respective total protein were not statistically significant in both breeds (Table 3). This datum supports the consideration of a normal intestinal absorption of β-carotene in both breeds.

Table 1. Biochemical metabolic profiles of donkeys (Asinara vs. Sardo) in blood serum collected during negative and positive photoperiods. Analyzed parameters involved in the nutritional assessment and potential impact on retinol metabolism are reported. Analyzed metabolites drop in the physiological range in both breeds

Breed	Asinara		Sardo	
Coat	Albino		Pigmented (gray)	
Photoperiod	Positive	Negative	Positive	Negative
Animals	6	17	6	17
Parameter				
Glucose (mg/dl)	38.8 ± 16.1	68 ± 7.91	53.0 ± 10.7	60.3 ± 4.84
Total protein (g/L)	82.3 ± 21.7	69.7 ± 2.61	84.0 ± 10.6	58.2 ± 7.27
Zinc (mg/dl)	40.6 ± 2.34	34.5 ± 2.12	44.1 ± 8.88	38.2 ± 2.19
Triglycerides (mg/dl)	67 ± 22.1	59.5 ± 17.7	69.3 ± 23.2	60.4 ± 17.7
Cholesterol (mg/dl)	78.8 ± 20.3	75 ± 2.82	83.6 ± 5.65	87.2 ± 7.59
Urea (mg/dl)	42.0 ± 13.4	38.1 ± 1.27	48.8 ± 11.5	31.2 ± 9.54
Lipase (U/L)	16.1 ± 1.31	14.5 ± 0.22	16.2 ± 4.12	18.0 ± 1.8

Table 2. Retinol levels (μg/ml) in blood serum of Asinara versus Sardo donkeys during different photoperiods (negative vs. positive). Values are expressed as mean and pooled standard error (SE). Retinol concentrations in serum clearly indicate the increase of circulating retinol in the bloodstream of Asinara white donkeys during the positive photoperiod, if compared with retinol concentrations in serum from same animals during the negative photoperiod. By contrast, Sardo donkeys do not show any variations of circulating retinol levels across different photoperiods. Negative photoperiod concentrations of retinol from Asinara white donkeys are slightly higher than those determined in blood serum of Sardo donkeys

Photoperiod		Positive	Negative	
	Animals (n)	23	23	
Breed				**SE**
Asinara	6	0.738[a]	0.522[ab]	0.04
Sardo	17	0.486[b]	0.492[b]	0.03

Values that do not share a letter are significantly different ($p < .05$).

Table 3. Correlation coefficients and p-values between circulating retinol, zinc (Zn), and total protein (TP) in Asinara white donkeys. No statistic significance was pointed out with blood serum concentrations of Zn and TP, whereas a statistically significant positive correlation was found between Zn and TP circulating levels

Correlations p-value	Retinol (μg/ml)	Zn (mg/dl)
Zn (mg/dl)	−0.276	
	0.172	
TP (g/L)	−0.294	0.670
	0.137	0.000

4. DISCUSSION

Herbivores fed on natural diets cannot consume adequate amount of retinol, necessary to cover their nutritional requirement, but they have to operate a conversion from its pro-vitaminic form (β-carotene) in the diet, from vegetal sources. As previously said, about 600 different compounds can be accounted in nature, of which only 50 can be found in human and animal diets. In particular, carotenes are hydrocarbons, thus their chains are only composed by C and H. Carotenoids containing an unsubstituted β-ring and a C11 polyene chain are termed provitamin A, and they can display biological activities, once enzymatically converted in the animal body. Provitamin A carotenoids from plants are important sources of dietary vitamin A, or retinol, for herbivores; they can be found primarily in fresh vegetables and in some particular fruits. Bioaccessibility of β-carotene from fat digestion, and its bioavailability, following the conversion into retinol, is genetically ruled (enzyme-dependence) [28, 29, 30]. Moreover, nutritional deficiencies of iron, zinc, and protein may also affect conversion rates of β-carotene into vitamin A. Therefore, the bioavailability of retinol depends on the genetic type of individuals and on overall nutritional-metabolic status. From a strict nutritional viewpoint, this is translated into a variable capability to digest, adsorb, and convert β-carotene into retinol. The retinol can be later acquired by organs and tissues and stored in liver as retinyl esters (stellate cells mainly), retina (in the conversion of retinol–retinal–rhodopsin and reverse, for the visual function), fat and skin [31]. Previous studies in epithelial cells from skin of rats have shown that dietary retinoic acid supplementation induces transglutaminase activity, being this enzyme involved in programed

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cell death, and maybe involved in the inhibition of carcinogenesis [32]. Intestinal conversion of β-carotene to vitamin A decreases when an experimental oral dose of β-carotene increases [33]. This can be considered as a safe biological way to protect against the risk of the fat soluble vitamin A excesses, accumulated in tissues and organs, like the liver. In fact, it was seen that despite high intakes of β-carotene, retinol levels do not increase proportionally [33, 34]. However, β-carotene can be converted to retinol with different efficiency rates in the diverse animal species [34]. To the best of our knowledge, the conversion efficiency of β-carotene into retinol was not experimentally determined in the donkey, but our results suggest that this species might be an efficient converter. It is known that different cleavage sites of β-carotene molecule may give rise to diverse biochemical pathways, depending on symmetric cleavage by β-carotene-monooxygenase (β,β-carotene15,15'-monoxygenase 1, BCMO1), or eccentric cleavage operated by β-carotene-dioxygenase (β,β-carotene-9',10'-dioxygenase, BCDO2) [35]. Symmetric or eccentric cleavages give rise to a series of products from βcarotene molecule, with diverse biological activities [34]. Thus, it is established that β-carotene molecule does not produce retinol only [34]. Results obtained from this trial seem to suggest that both Asinara white and Sardo donkeys are efficient converters of β-carotene into retinol. It could be argued that Asinara white donkeys may intake higher amounts of β-carotene with the diet, by a more accurate selection of naturally available plant species. However, this aspect does not appear to be plausible given the nutritional status of animals from both breeds, which appeared similar throughout the experimental period. The intake of β-carotene implies the consumption of proportional dietary fat with the diet, that would have led to different energy intake and consequent energy storage. Additionally, body condition scores together with variations associated with circulating total triglycerides in the bloodstream would have varied accordingly, but this was not found to differ between the two breeds. It was therefore considered that the metabolic response in the albino donkey can be elicited by increasing natural daylight exposure, namely during positive photoperiod. In fact, circulating retinol levels resulted higher in blood serum of Asinara white donkeys when compared to those determined in Sardo ones, during increasing intensity and duration of exposure to natural light. Such finding can be related to the key biological functions of vitamin A (retinol) and its aldehyde (retinaldehyde) in the visual function. The whole

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biochemical process leading to the involvement of retinol in the formation of rhodopsin is not reported here, as extensively reviewed by Palczewski (2006) [36]. Despite a comparable allowance to dietary β-carotene from natural feeding sources available in the environment, Asinara white donkeys could efficiently mobilize retinol from tissue stores producing high circulating retinol levels in the bloodstream. This datum is supported by the circulating levels of retinol in Asinara donkeys during the negative photoperiod, comparable to the average levels observed in Sardo donkeys throughout the year. At Sardinian latitude, the month of June represents the culmination of the positive photoperiod with a maximum of daylight duration of nearly 15 hours/day. This was associated with the fact that, under comparable conditions of dietary β-carotene from naturally available vegetation, Asinara donkeys display higher levels of circulating retinol than Sardo breed donkeys do, which can, however, rely on melanin for photoprotection. Dietary β-carotene is consumed normally with fat compounds of the diet. Thus, β-carotene follows dietary fat digestion and absorption processes. In the herbivore, the pro-vitaminic β-carotene is absorbed with vegetal fats in the small intestine. In particular, the absorption of β-carotene from mixed micelles in the chymus of the small intestine occurs in the brush border of the enterocyte (Figure 3)

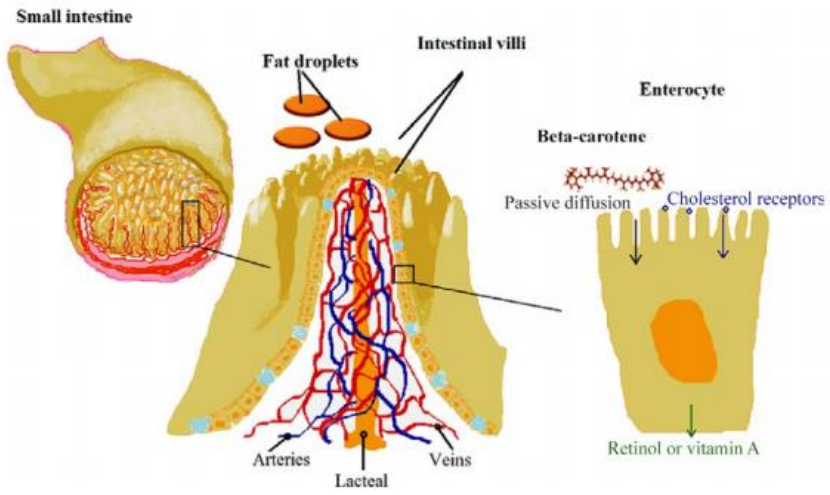

Figure 3. Scheme of β-carotene fate as precursor to retinol (Vitamin A).

The conversion is operated in the brush border of the enterocyte of the small intestine. Circulating β-carotene around the limit of sensitivity suggests that donkeys are efficient converters of the provitamin into retinol. Blood serum concentrations of retinol turned out to differ in Asinara versus Sardo donkeys during positive photoperiod, as an adaptive metabolic measure to overcome the lack of melanin in specimens of albino breed. Retinol

[36] K. Palczewski, Annu. Rev. Biochem., 75 (2006) 743–767.

levels are suggestive of a pulsed mobilization of retinol into the bloodstream from liver stores in Asinara white donkeys.

β-Carotene absorption can follow two fashions, but the detailed mechanisms ruling on which pathway may be preferable is not elucidated to date. Indeed, the literature reports [37, 38] that BCOD2 rapidly metabolizes nonproteinoid carotenoids. However, it is well known that β-carotene is not fully converted into retinol and that the conversion is self-modulated according to the level of retinol already synthesized [28]. One mechanism of absorption is represented by the passive diffusion of β-carotene through the mucosal layer of the intestine into the vasum chyliferum, which conveys fatty nutrients in the lymphatic circulation. Alternatively, the absorption can occur through cholesterol receptors expressed on the cell membrane of the brush border of the enterocyte [28]. In addition, interactions with other nutrient levels may impair β-carotene absorption. The literature reports that nutritional deficiencies of iron, zinc, and protein may also affect estimates of the vitamin A equivalency of β-carotene [28]. As to elements, Iron deficiency disrupts retinol homeostasis and results in decreased mobilization of vitamin A from the liver and low serum retinol concentrations in rats [39]. Marginal zinc deficiency results in a significant reduction in β-carotene absorption in rats [40] and may also limit production of retinolbinding protein and interfere with retinol homeostasis. Indeed, protein deficiency too is associated with reduced intestinal conversion of β-carotene to vitamin A in rats [41] and may interfere with production of chylomicrons, lipoproteins, and retinol-binding proteins, with potential impacts on retinol metabolism [28, 39, 40, 41]. In both breeds, no nutrients were found to be below the minimum level of the physiologic range. Moreover, in Asinara white donkeys, no statistically significant correlations with retinol levels and zinc or total protein concentrations in blood serum were found. The comparison between the overall conditions observed in both groups of animals allowed us to draw several conclusions about the nutritional assessment, supported by nutrition-related metabolic profiles. As a matter of fact, nutritional deficiencies with a direct impact on coat and skin health may involve polyunsaturated fatty acids and fat soluble vitamins, [31, 32]. The high concentration of retinol in the bloodstream during positive photoperiod can be due to the increased mobilization of retinyl esters stored in the liver.

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However, as neither intestinal nor hepatic mechanisms of retinol biosynthesis or mobilization were investigated in Asinara white donkeys, it is assumed that in vivo circulating retinol levels reflect the need to restore hematological levels in case of augmented need of photoprotection. The photoperiodism can strongly influence some important physiological functions of animals, in particular of wild and feral animals. For example, the mechanism behind the stimulus in relation to photoperiod and the retinal stimulation by daylight is capable to modulate the neuroendocrine retinal-pineal-gonadal axis. That way, cyclic reproduction of many animal species is influenced under seasonal control. In a similar way, though with different goals, retinol levels in the bloodstream of albino donkeys of Asinara breed can be modulated by photoperiodism, via stimulation of a more susceptible retina to natural light intensity and duration, in order to guarantee photoprotection of exposed tissues.

5. CONCLUSIONS

Higher blood serum concentrations of retinol in Asinara donkeys (albino breed) were found in relation to positive photoperiod, than found in blood serum from donkeys of Sardo breed (grey coated) involved in this trial. The concentration of this nutrient-related metabolite can represent an alternative way to the lack of melanin in tissues, to explain the adaptation of albino donkeys in the natural Mediterranean environment. In this case, retinol may be an adaptive metabolic key to overcome the higher susceptibility to sun radiation of albino animals. Surprisingly, this peculiar form of albinism extended to all individuals of Asinara breed appears adapted to the environment. Probably, photoprotection might be achieved through higher levels of available retinol in the bloodstream capable to reach peripheral tissues. In vivo determination of high serum concentration of retinol in the Asinara donkeys paves the way to further investigations on the specific pathways leading to unpigmented skin protection from exposure to sun radiation.

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CONFLICT OF INTEREST

Authors declare no conflict of interest exists.

Chapter 2

Levels of LDH and CPK vary in blood serum of Asinara donkeys (albino) vs. Sardo donkeys (pigmented) in presence of similar circulating α-tocopherol

Levels of LDH and CPK vary in blood serum of Asinara donkeys (albino) vs. Sardo donkeys (pigmented) in presence of similar circulating α-tocopherol

M. G. Cappai, F. Accioni, G. Biggio, R. Cherchi, G. Boatto, W. Pinna
Submitted to The Veterinary Journal.

ABSTRACT

Alfa-tocopherol possesses marked antioxidant properties within the group of fat soluble vitamin E found in plants. Free grazing herbivores can benefit from the large abundance of feed sources in the environment, naturally rich in α-tocopherol for antioxidant properties. However, the phenotype of a worldwide unique acknowledged breed of albino feral donkeys living in the natural reserve of Asinara Island Park (Italy) may represent a challenge for health maintenance. The metabolic profile was comparatively investigated, with particular regard to circulating α-tocopherol baseline levels in specimens of Asinara (albino) vs. Sardo (pigmented coat) breeds. Circulating α-tocopherol found in the bloodstream of Asinara vs. Sardo donkeys under free grazing conditions at the peak of the positive photoperiod turned out to reach similar values (2.114 vs. 1.872 μg/ml, respectively, $p=0.676$). Interestingly, significant differences were instead observed as to circulating lactate dehydrogenase (LDH, $p=0.022$) levels, in association with increased creatine phosphokinase (CPK, $p=0.076$), both higher in the totality of Asinara donkeys. In the horse, the combination of increased values in the bloodstream of both such enzymes can be referred to muscle damage and, if coupled with low dietary vitamin E, can outline syndrome from dietary vitamin E deficit. Despite all the donkeys appeared healthy and showed comparable aspartate aminotransferase levels (AST, 254±52 vs. 296±133 U/l respectively, $p=0.405$), a potential subclinical disorder in albino specimens involved in this trial may be supposed, posing the question about α-tocopherol requirements in Asinara donkeys for antioxidant purposes.

KEYWORDS Albinism; Mediterranean maquis; Myopathy; Sun radiation; Vitamin E

HIGHLIGHTS

- Feral Asinara donkeys display OCA1 albinism and sun radiation may challenge survival rates.
- Natural feed rich in vitamin E maybe be preferably selected in nature for antioxidant properties.

- Similar circulating α-tocopherol levels were found in Asinara vs. Sardo (pigmented) donkeys.
- Circulating CPK and LHD were above the upper limit of the normal range in Asinara donkeys.
- The adequacy of α-tocopherol levels of 2 μg/ml in donkeys should be tested on phenotype.

1. INTRODUCTION

Browsing and grazing animals can intake large amounts of Vitamin E from fresh feeding sources available from spontaneous vegetation. Vitamin E accounts a group of fat soluble compounds involved in several biological processes in the animal body, important for health maintenance [1].Alfa-tocopherol represents one of the most biologically active forms of vitamin E group [2, 3]. Isomers of vitamin E cannot be synthesized de novo in the animal body, thus circulating α-tocopherol determined in the bloodstream of animals derives from the diet. It was established that tocopherols are the most abundant isoforms of Vitamin E in leaves, whereas tocotrienols (among other isoforms of Vitamin E) are chiefly found in seeds [2]. Though tocopherols can be found in plant seeds too, γ-tocopherol is abundantly synthesized, whilst α-tocopherol only residually. Levels of circulating α-tocopherol in the bloodstream of free ranging animals may reflect leaf-based natural diets, especially for grazers, like equines.

The content of natural α-tocopherol dramatically decreases during feed processing, due to intrinsic lability (light and heat/cold sensitive) of such chemical compound. In addition, vitamin E synthesis in plants broadly varies, according to plant species and season (higher during spring-summer than during fall-winter) [4]. The daily requirement of Vit E (1-2 mg α-tocopherol/ kg BW in a 500 kg BW horse in light work, NRC 2007) may not be adequately met if the horse is fed on a hay-based diet, unless purposely supplemented. In particular, the biologic role of vitamin E in the horse has been recently reviewed by Finno and Valberg (2012) [1]. Same authors pointed to the activity of α-tocopherol, often combined with Se, acting as scavenger of reactive oxygen species (ROS) in support to endogenous antioxidant systems of the horse. In the last decades, a number of evidence based reports pointed to the onset of clinical symptoms due to chronic deficiency of vitamin E in the diet of stabled

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horses. Disorders may involve musculoskeletal tissues or lead to neurodegenerative syndromes, often depending on individual's age and breed. Vitamin E deficiency in foals and adult horses, with or without chronic Selenium intakes below requirements, appeared to be correlated with various clinical signs [5, 6, 7, 8, 9, 10, 11, 12].

As to the donkey, only few contributions report detailed effects of the dietary regime on biochemical profile and clinical overall conditions, with emphasis on specific nutrient deficiencies [13, 14, 15, 16, 17, 18]. Actually, very little is known about reference intervals of circulating parameters for this species [16, 19, 20, 21, 22, 23, 24, 25, 26]. Baseline levels of α-tocopherol are unavailable for the grazing donkey in the present literature, to the best of our knowledge.

It was hypothesized that the donkey might be susceptible to disorders from dietary vitamin E deficiency, like the horse. In particular, the effect of the albino phenotype in a worldwide unique acknowledged breed of feral donkeys freely grazing in the National Reserve of Asinara Island Park appeared worthy of investigation. In view of the natural feeding habits of

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feral donkeys, α-tocopherol deficiency may not be expected to involve free grazing animals in the wild, but the peculiar condition (albinism) of all specimens of Asinara breed at Mediterranean latitudes, during the peak of the positive photoperiod (the month of June in the boreal hemisphere) was supposed to represent a metabolic challenge. In albino donkeys, the excess of sun exposure could play a role in the perturbation of the homeostasis in which endogenous antioxidant systems may find a support in dietary biologically active compounds with antioxidant properties, like α-tocopherol. The skin is the largest organ of the animal body and is intensely exposed to the action of environmental factors. Melanin represents the natural pigment of skin, hair, iris and natural opens, produced by melanocytes to protect tegument cells from UV damage. To such an extent, albinism in wild and feral animals may represent a conditioning factor behind the selection of feed rich in antioxidant compounds while grazing under UV exposure. If this is the case, it could be argued that albino Asinara donkeys may prefer leafy feeding sources rich in α-tocopherol, in the attempt to overcome the augmented requirements of natural antioxidant compounds.

The aim of the present study was to determine the circulating levels of α-tocopherol in the bloodstream of Asinara in comparison with Sardo donkeys (pigmented, grey coat), in the month of June, during the peak of the positive photoperiod. In addition, the metabolic profile in all specimens of both breeds was comparatively explored for screening organ functions (liver, kidney and pancreas) and overall health conditions.

2. MATERIALS AND METHODS

2.1. Location of the trial

This study was carried out on Asinara island (N 41° 4' 0.012", E 8° 16' 0.012", 51.9 km^2 in Sardinia, Italy), established as a National Park (Official Gazette of Italian Republic, 1997) and Marine Reserve (Official Gazette of Italian Republic, 2002) of the Autonomous Region of Sardinia (Italy), in the Mediterranean Sea (Figure 1). The park is extended over an area of 51.9 km^2, covered by Mediterranean maquis. Albino donkeys of Asinara owe the name of the breed to the island, where they live in the wild since centuries [27]. The population of Asinara donkeys on the island is currently estimated to account 140 individuals (Antonelli, 2017 personal communication). A lesser number of Asinara donkeys is distributed to other parks and reserves of the Autonomous Region of Sardinia, while few animals are kept in the rest of Italy (specimens: 294; conservation status: critical; records of the National Registry of Local Minor Equine Breeds, Association of Italian Breeders, 2016). Asinara donkeys share

[27] F. Cetti., I Quadrupedi di Sardegna. In: G. Piattoli (eds) I Quadrupedi di Sardegna. G. Piattoli, 1774.

the territory with other wild animal species of autochthon fauna. Feral Sardo breed donkeys (Figure 2) live on the isle of Asinara as well and live in small groups with Asinara donkeys.

Figure 1. Map of Sardinia and latitudes of Asinara island (yellow) where the National Park and Marine Reserve of Asinara is established.

Figure 2. Jenny and foal of Asinara breed in the natural Mediterranean maquis of Asinara Park.

2.2. Animals and enrollment criteria

The investigation involved a total of 23 adult donkeys (age: between 5 and 6 years, established from records of the Park), of which 11 were stallions and 12 jennies. Asinara (n= 6) and Sardo (n= 17) breed donkeys were enrolled to achieve similar sex ratio (1:1). All animals were electronically identified (EID, EU Regulation 2015/262) and recorded in the Official Register of the albino donkey of Asinara and Sardo breeds (Ministerial Decree 27/7/1990), respectively. The proportion of specimens from each breed was established to be representative of populations of Asinara and Sardo donkeys, namely hundreds and thousands under natural condition, respectively. Number of heads is checked and updated every year. Sanitary surveillance is also carried out by the Veterinary Services of the local District, for the control of equine infectious diseases. Wild and domestic equines undergo serological tests (Coggins test, mandatory for horses, donkeys, mules and hinnies). On blood sampling for serological analysis, one serum aliquot was used to determine basic biochemical profile of all donkeys enrolled. In the light of the focus of this investigation, circulating α-tocopherol was also determined on same samples, without requiring further manipulations of animals. Blood sampling coincided with the peak of positive photoperiod (month of June 2017, after three months of increasing light hours per day). Each donkey had free access to same natural areas.

On blood sampling, donkeys underwent the nutritional assessment according to Cappai et al. (2013) [14].

Whole blood was collected through the puncture of the jugular vein. For this purpose, all animals were gathered in a paddock with mobile fences, by the personnel of the Park. Animals were induced to step into a corridor with the use of mobile fences, leading to a horse stock. All animals underwent a same protocol and were manipulated in respect of animal welfare, for the sole moment needed for blood sampling and EID code checking. All animals were immediately released, when all procedures were terminated.

Individual tubes were covered with tin-foils to protect blood from light and classified with individual labels. Tubes were held in the upright position through polystyrene cases and kept in a refrigerated bag used to transport samples and assure adequate temperature during the transfer to the laboratory.

2.3. Analytical protocols and methods

All laboratory procedures were started within 6 hours of collection. In field and laboratory protocols for collection, storage and analyses of blood samples were carried out in the dark,

to avoid photo-degradation of α-tocopherol. Each individual serum was screened to explore organ function and assess overall metabolic conditions. Prior to chemical analyses, samples were centrifuged at 1500 g for 10 minutes. Two aliquots (2 ml) of each serum sample were stored in sterile vials and frozen at -20°C until further analysis. All the samples were analyzed within one week, through an automatic light-protected biochemical analyzer (Mindray BS 200, Shenzhen, China). The assessment of organ function and overall health conditions (liver, kidney, pancreas and skeletal muscle) consisted in the determination of serum concentration of ubiquitous intermediate metabolites enzymes, nutrients and macro-minerals (alanine transaminase, ALT; aspartate aminotransferase, AST; gamma-glutamyl transferase, γ-GT; creatinine, CREA; Urea; total protein, TP; total cholesterol, Cho; total triglycerides, Tri; amylase, Amy; Lipase, LIPA; lactate dehydrogenase, LDH; creatine phosphokinase, CPK; calcium, Ca; phosphorus, P).

For the determination of α-tocopherol, high pressure liquid chromatography coupled with an ultraviolet detector (HPLC-UV) was carried out. All standards and solvents were purchased from Sigma Aldrich (Milan, Italy). Stock solution (1 mg/ml) of α-tocopherol was prepared in chloroform/methanol (50/50). For the calibration curve, standard stock solutions were diluted with methanol and kept frozen at -20°C, protected from light. Serum level of α-tocopherol was measured at 280 nm. Chromatographic separation was carried out on a Waters Symmetry C18 column (4.6 x 150 mm, particle size 5 μm, Waters, Milford, Massachusetts). The injection volume was 20 μl. The mobile phases used were acetonitrile/methanol/Milli-Q water (64.5/33/2.5) at 1 ml/min. Data were acquired and processed by Breeze Software (Waters, Milford, Massachusetts).

Samples were prepared as follows: 0.3 ml of serum was vortexed with 0.6 ml of acetonitrile and centrifuged at 3500 g at 4°C for 10 min. The supernatant was dried under a stream of nitrogen and the residue was reconstituted in 0.15 ml of mobile phase [28, 29, 30, 31, 32].

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2.4. Analysis of data and statistical methods

Circulating levels of metabolites and baseline concentrations of α-tocopherol in the blood serum of donkeys were analyzed and interpreted according to species-specific reference values available in the present literature. Data were analyzed by Student t-test, for the comparison of averages determined in specimens of the two breeds (Asinara vs. Sardo). The statistic significance was set for p-value<0.05.

3. RESULTS

A total of 23 serum samples was collected from 23 donkeys in the month of June. All animals involved in the trial appeared healthy. No specific clinical signs could be pointed out in specimens of both breeds, except for skin redness involving ear tips, ocular contour (showing signs of epiphora) and backline in all Asinara donkeys, considered as common findings for the breed. Adequate body condition (BCS, based on a 5-points scale, 1=emaciation to 5=obesity) was scored in both in Asinara and Sardo donkeys (3.25±0.15 vs. 3.50±0.10, respectively).

Biochemical profiles of specimens enrolled in this screening trial did not point to significant differences between breeds. Metabolites explored for organ function dropped within the physiological range for the species (data not shown), except for lactate dehydrogenase (LDH p=0.022) and creatine phosphokinase (CPK p=0.076), as reported in Table 1. Alfa-tocopherol concentration in the blood serum appeared similar in both breeds. Aspartate aminotransferase (AST) did not display to vary between breeds and values were within the physiological range for the species [25, 26]. Table 1 summarizes results. In Figure 3 mean values and SD of LDH and CPK with respective reference ranges in the two breed are displayed.

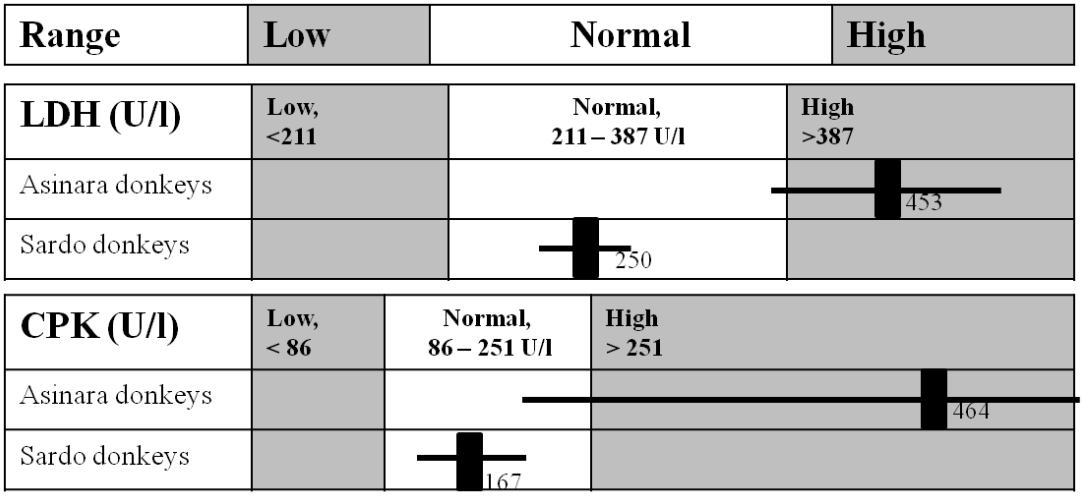

Figure 3. Reference range and average concentration of LDH and CPK in the blood of Asinara (albino) vs. Sardo (pigmented).

Table 1. Biochemical metabolic profiles of donkeys (Asinara vs. Sardo) in blood serum collected during positive photoperiod. Analyzed metabolites depict the peculiar metabolic similarity of α-tocopherol levels meanwhile underlying the increased levels of LDH and CPK in Asinara exclusively.

Breed **Coat** **Photoperiod** **Animals** **Parameters**	**Asinara** **Albino** **Positive** **6**	**Sardo** **Pigmented** **Positive** **17**	**Significance** **p-value**
α-tocopherol (μg/ml)	2.11±0.52	1.87±0.70	n.s.
Triglycerides (mg/dL)	71.2±19.1	61.3±19.7	n.s.
Cholesterol (mg/dL)	74.3±20.3	76.1±2.82	n.s.
Lipase (U/L)	15.9±1.24	16.1±0.98	n.s.
AST (U/L)	254±52	296±133	n.s.
LDH (U/L)	453±122	250±34.5	0.022
CPK (g/L)	465±274	167±30.8	0.076

4. DISCUSSION

Albinism in wild animals may represent a serious health risk under excess of sun exposure [33]. By contrast, the description of the presence of Asinara donkeys on the homonymous island dates back to the 18th century [27]. Despite the critical status of conservation, it could be reasonably postulated that alternative solutions to damage and oxidative stress may help to explain the adaptation to the Mediterranean environment. A possible strategy for albino

[33] J. Prado-Martinez, I. Hernando-Herraez, B. Lorente-Galdos, M. Dabad, O. Ramirez, C. Baeza-Delgado, C. Morcillo-Suarez, C. Alkan, F. Hormozdiari, E. Raineri, J. BMC genomics, 14 (2013) 363.

grazers in the wild may consist in an increased intake of feeding sources rich in natural antioxidants, like α-tocopherol. Both carotenoids and tocopherols are reported to be the most abundant groups of lipid-soluble antioxidants in chloroplasts of vegetal cells. Such compounds are prominently synthesized in photosynthetic tissues of the plant when oxygen toxicity and lipid peroxidation should be contrasted to reduce the effect oxidative stress from excess of light and heat.

The comparison of α-tocopherol levels in the bloodstream of animals of the two breeds contributes to reinforce the hypothesis that a metabolic adaptation may be a reasonable strategy for the survival of Asinara donkeys in the wild Mediterranean environment. Previous phylogenetic analyses [34, 35] suggested that the fixation of the mutation for albinism has been favored by geographical isolation. The autochthon origin of Asinara breed was supported by the molecular analyses carried out by Pinna et al. (1998) [34] who reported a common ancestor for Asinara and Sardo donkeys and the possibility that albino donkeys branched out of the autochthon pigmented Sardo donkey. Cosseddu et al. (2001) [35] supported these results in later investigations.

The rationale behind this trial moved from the results obtained in previous trials [14, 15, 36]. The very efficient conversion of dietary precursors and body stores into circulating retinol in the Asinara donkeys if compared to Sardo donkeys was pointed out in own recent researches. Such results pointed to a metabolic adaptation of Asinara donkeys to the natural environment and seasonal feeding sources, potentially driven by the oculo-cutaneous albino form of type 1 (OCA1). Against this background, the similar levels of circulating α-tocopherol found may be suggestive that both breeds rely on same plant parts and may share feeding habits. However, the combination of increased concentrations of circulating LDH and CPK, may be suggestive of potential muscle damage. There is general consensus of the correlation between circulating values of α-tocopherol below adequacy (2 μg/ml) and higher risk of myopathy and neurodegenerative disorders for the horse, though a linear effect on the onset of clinical signs is still under debate.

No specific health problems were found in Asinara donkeys if compared to Sardo donkeys under same grazing conditions. In general, wild animals are aware grazers in nature and can wisely select feeding sources to avoid toxic or harmful plant species. Captive animals or domestic livestock may be untrained to anti-pastoral or anti-nutritional traits of plants in

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natural pastures [37]. Potential xenobiotic ingestion leading to photosensitization in Asinara donkeys cannot be excluded but represents a remote explanation to skin redness and the epiphora observed.

The variations of circulating levels of LDH and CPK in the two breeds of donkeys are suggestive of homeostasis perturbation, though no syndrome from deficient vitamin E to adequately cover requirements in the donkey was described before. The hypothesis that albino donkeys may be prone to metabolic perturbation can be here supported by the increase of those enzymes found exclusively in apparently healthy Asinara donkeys, against the background of comparable circulating levels of α-tocopherol found in both breeds.

5. CONCLUSIONS

In the light of results obtained in this trial, similar circulating levels of α-tocopherol between Asinara and Sardo donkeys seem to point to similar feed selection available in the wild, during positive photoperiod in the National Park of Asinara at Mediterranean latitudes. Despite apparently healthy, the combination of increased levels of LDH and CPK enzymes may highlight subclinical conditions of homeostasis perturbation, related to muscular tissue involvement (cell membrane instability in the albino donkey?). The definition of physiological ranges of circulating α-tocopherol may be useful to identify reference intervals associated with the clinical condition of the donkey, to establish adequate or deficient dietary supply. In the albino feral donkey of Asinara breed, circulating α-tocopherol in the blood serum around 2 μg/ml appears suggestive of the strong influence of the phenotype on the concomitant LDH and CPK increase, not observed in the Sardo donkey.

CONFLICT OF INTEREST STATEMENT

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Chapter 3

Supplementation of α-tocopherol/selenium in the diet of breeding stallions during negative photoperiod. Part I: effects on semen quality.

Supplementation of α-tocopherol/selenium in the diet of breeding stallions during negative photoperiod. Part I: effects on semen quality.

M.G. Cappai, A. Taras, I. Cossu, R. Cherchi, C. Dimauro, Francesca Accioni, G. Boatto,D. Gatta, W. Pinna

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ABSTRACT

Dedicated nutritional strategies may help to improve semen quality of breeding stallions. However, effects from dietary α-tocopherol in association or not with selenium supplementation are still under debate and limited literature reports contrasting results. The present investigation aimed to test the effect of α-tocopherol/Se supplementation in the diet of breeding stallions on semen quality during negative photoperiod to maximize the diet effect modulation, if any. The trial involved 10 stallions serving in a same stud centre. Stallions were allotted to two groups, consisting of 5 horses each. On adaptation, horses were fed with a same diet, based on concentrate feed (Crude Protein: 14%; Crude Fibre: 9.5%; Crude Fat:4.0% Crude ash: 7.8%; Cu: 40 mg/kg; Zn: 120 mg/kg; all rac-tocopheryl-acetate: 40 mg/kg as fed) for horse offered up to 0.9% DM/BW/d and good quality hay for 10 days, assuming a maximum daily DM intake of 2.5% of BW. One group (TG) switched to the experimental diet (additional 200 mg all rac-tocopheryl-acetate + 0.18 mg SeMeth/100 kg BW/d, mixed with the pelleted feed, as fed) for eight weeks, while the non-supplemented group (CG) continued to be fed with the same identical pelleted diet and hay. TG stallions ingested 204±10.4mg/100 kg BW/d of all rac tocopheryl-acetate, representing 4-folds of daily intake of CG stallions. Circulating levels of blood serum α-tocopherol resulted to differ significantly ($p<0.001$) in TG *vs.* CG stallions (3.22±0.19 *vs.* 1.01±0.04 μg/ml), respectively. Semen quality resulted to vary in relation to age. Horses younger than 15 years displayed better semen quality, independently from the dietary supplementation. Interestingly, a significant ($p=0.039$) reduction of the percentage of immobile spermatozoa in stallion semen could be observed at the interaction of high circulating α-tocopherol x high copper levels if compared to low α-tocopherol x low copper levels (21.1±1.09% vs. 41.1±4.54%, respectively). Circulating copper was negatively and weakly correlated ($\varrho = -0.136$; $p=0.358$) with horse age in a non significant way. α-tocopherol/Se supplementation at tested amounts in TG stallions below 15 years of age and with adequate levels of circulating copper appeared to have a positive impact on the reduction of the percentage of immobile spermatozoa.

ABBREVIATIONS:

AA, Anglo-Arab;

ADF, acid detergent fiber;

ADG, averaged daily gain;

ADL, acid detergent lignin;

ANOVA, analysis of variance;

BW, body weight;

CASA, computer assisted sperm analysis;

CFi, crude fiber;

CG, control group;

CP, crude protein;

DM, dry matter;

EE, ether extracts;

EU, European Union;

HPLC-UV, high pressure liquid chromatography coupled ultra violet;

ICMJE, International Committee of Medical Journal Editors;

Mil/ml, millions/milliliter;

NDF, neutral detergent fiber;

NfE, nitrogen free extract;

ROS, reactive oxygen species;

T_0, T_1, T_2, time0, time1, time2;

TG, treated group;

KEYWORDS Horse; Nutrition; Semen quality; Stud centers; Vitamin E.

1. INTRODUCTION

The conservation of desirable traits in sport horse breeding practices commonly accounts parental genealogy and body morphology predisposing to the athletic potentials of the future foal destined to equestrian disciplines. Enrolment of stallions in the studbook is commonly based on the genetic value of the horse, as well as on sport performance achieved during the athletic career, which not necessarily coincide with reproductive attitudes [1, 2, 3]. Several factors appear to contribute to maintain semen quality [4, 5] and, among such, nutritional strategies may play a role in improving semen quali-quantitative parameters. The individual management of the breeding stallion should therefore consider different goals altogether, in which adequate semen quality may represent an issue. It is well known that semen quality is not synonym of fertility [6, 7, 8, 9], but some semen characteristics may represent a reliable tool to estimate the reproduction potentials of the stallion. Concentration of spermatozoa per unit (Mil/ml) of ejaculated (gel free) may also represent a parameter for semen quality evaluation, potentially linked to fertility, though dramatic changes may be observed from day to day and not linked to nutritional management solely. In fact, despite to a lesser extent than what observed in mares, the stallion is susceptible to photoperiod for semen production, which appears to decrease during the negative one [3]. Among the quantitative and microscopic characteristics, the proportion between motile and immobile spermatozoa percentages also affects the chance for successful conception. As to motile spermatozoa, the "straightness" parameter is associated with higher chance for progressive movement towards the oocyte. At this regard, a computer-assisted sperm analysis (CASA) was developed to understand the movement of spermatozoa from fresh semen samples and currently diffused for semen quality assessment [10, 11, 12]. Note of worth, the high metabolic activity and the consequent energy needs of spermatozoa play a pivotal role as to survival rates outside the male reproduction apparatus. The relevant presence of reactive oxygen species (ROS) is

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reported to be among the key factors for membrane damage of the sperm cell. Arguably, lipid peroxidation may in fact contribute to low fertility rates of stallions due to the presence of high percentages of immobile spermatozoa. Oxidative stress can impair the survival rate of the sperm cell, in which unsaturated fatty acids content in cytoplasm is naturally high. Thus, it could be hypothesized that exogenous (dietary) substances may behave as scavengers taking part in antioxidant systems for the modulation of the oxidative status of sperm cells. Recently, Finno and Valberg (2012) [13] reviewed the several biological activities of vitamin E isoforms and report the target body systems in which α-tocopherol in particular can take part. Indeed, vitamin E was acknowledged as the vitamin of fertility, but this property was assessed in experimental trials in rats [14]. As to exogenous antioxidant substances which may be involved to contrast the detrimental effects of ROS, dietary compounds may be used as scavengers and therefore contribute to modulate the oxidative - antioxidative status of active spermatozoa. Despite the literature appears plenty of contributions highlighting the effects of different groups of active substances directly on metabolism of spermatozoa *in vitro* for semen quality preservation of cryoconservation (including vitamin E), only few authors carried out experimental feeding trial for the nutritional management of stallions to test the diet effect modulation on semen quality. At this regard, controversial results are reported at present to the best of our knowledge [15, 16, 12, 17].

The present study aimed to test the effect of the supplementation with synthetic α-tocopherol in association with Se in the diet of breeding stallions. It was aimed also to test the dietary effect during the negative photoperiod in order to maximize the effects from dietary supplementation on semen parameters. In this first part, result on overall quality of semen from supplemented breeding stallions is reported.

2. MATERIALS AND METHODS

2.1. Animal care

Animal handling complied with the recommendations of European Union Directive 2010/63/EU concerning animal care. All procedures reported in this trial belong to conventional clinical practices; in particular, blood and semen sampling, were carried out by

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expert veterinary practitioners and trained technicians. This article complies with the recommendations for the conduct, reporting and publication of scholarly work in medical journals ICMJE/2006.

2.2. Experimental design

A total of 10 stallions (Anglo-Arab breed, AA; Body mass: 510-530 kg; Age: 4 to 21 years-old) was enrolled in the trial. All animals involved in the study served as breeding stallions in a same stud centre, belonging to the Agency for Horse Breeding and Research of the Autonomous Region of Sardinia. Inclusion criteria of animals in the trial took into account: a) same breed; b) serving frequency during the previous stud season (three times a week, on alternate days); c) good health conditions and compliance with mandatory prophylactic measures for prevention of infectious diseases.

The experimental period matched with the negative photoperiod of boreal hemisphere, during the months of October and November 2016. All animals underwent same rearing and feeding conditions prior to the experimental feeding.

After an adaptation period of two weeks, in which all animals were fed a same basic diet and were sampled for semen every at 0, 24, 48 hours on every 3 days, the 10 stallions were allotted to two groups consisting of 5 stallions each. One group (TG) switched to the experimental diet, consisting of the supplementation with α-tocopherol and Se in granular form mixed with the pelleted feed and administered for 8 weeks. The other group continued to be fed with the basic diet and represented the control group (CG).

2.3. Blood and semen sampling schedule

At the beginning of the trial, (T_0 phase, adaptation period), at mid-experiment (T_1 phase), and at the end of the experimental feeding trial (T_2 phase), all horses were sampled for blood and semen; in addition, each horse was clinically inspected and assessed for body condition scoring (1-9 points-scale), according to Henneke and co-workers (1983) [18].

2.4. Experimental phases

Until T_0 phase, the totality of horses were raised and fed under same conditions. During the previous stud season (mid of February-end of June 2016), all stallions served three times a week each, on alternate days. All the stallions involved in this trial are athlete horses retired from racecourses, of high genetic value and successful sport performance.

[18] D.R. Henneke, G.D. Potter, J.L. Kreider, B.F. Yeates, Equine Vet. J., 15 (1983) 371-372

2.5. Dietary regime and feeding practices

Hay and concentrate were offered to stallions assuming a 2.5% of BW of daily DM intake, subdivided in 1.6% and 0.9%, respectively, like expected during stud season. Daily administration of hay was scheduled at 6:00 am and at 2:00 pm. The chemical composition of hay is reported in Table 1. Concentrate feed was a complementary pelleted feed for horses. Daily administration of concentrate feed was scheduled at 11:00 am and at 5:00 pm. Supplement in a granular form was mixed in the experimental diet of the TG with the pelleted feed, at the amount of 100 g /500 kg BW/horse, as fed. Such amount was mixed to provide additionally 200 mg all rac α-tocopherol, 18 mg Selenium/Methionine and 114 mg Zinc hydrate/100 kg BW/horse. The chemical composition of hay and mixed concentrate is reported in Table 1. During the last two weeks, daily concentrate intake was calculated by weighing concentrate offered and leftovers.

Table 1. Composition of analyzed nutrients of hay and mixed concentrate administered daily throughout the trial

Main nutrients (g/kg DM) and energy (MJ/kg DM) content	Hay (oat, ryegrass, clover)	Mixed concentrate
Dry matter (g/kg, as fed)	887	880
Crude Ash	135	7.8
Crude protein	149	140
Ether Extract	21.6	40.5
Acid detergent fibre	342	214
Neutral Detergent fibre	585	533
α-tocopherol (mg/kg DM)	9.8	44.1

*Supplement was added to concentrate administered to stallions of the TG, at a daily amount of 100g/500 kg BW per horse.

2.6. Laboratory analyses of feeds, whole blood, blood serum and semen

Feeds and leftovers were oven-dried (103 °C) and then ground (0.5 mm); samples were analyzed in duplicate according to Weende analysis described by Naumann and Bassler (2004). The crude protein content was determined using the Dumas combustion method.

The ether extract was determined by the Soxhlet apparatus. Fibrous fractions were determined according to Van Soest method (1991) [19].

Blood samples were taken at each control (T_0, T_1, T_2) for the determination of complete haematological and biochemical profiles. Each horse was sampled twice a day to take into account potential circadian biases on biochemical parameter fluctuations in the statistical analysis [20]. A first blood sample was scheduled at 8:00 am, 2 hours after hay administration, for whole blood collection through the puncture of the jugular vein. A second blood sample was scheduled at 7:00 p.m. Blood sample collection followed this protocol at each control throughout the study. Samples were cooled and tubes stored in polystyrene cases in the upright position into a cool bag, to grant adequate temperature during sample transfer to the laboratory. All samples were identified with horse's name, electronic individual code (EIC) and date of sampling and processed within 6 hours from collection for haematological profile. From whole blood stored in ethylenediaminetetracetic acid (EDTA) containing tubes, hematologic profile was determined through an automatic analyzer (Mindray BC-5000 Vet, Alcyon, Italy), with own reference intervals. Individual serum was screened for complete biochemical profile. Gel tubes with a clotting accelerator were used. Samples underwent centrifugation at 1500 g for 10 minutes. The serum was removed and stored in vial (2 ml) and frozen at -20 °C until analysis. Two sample aliquots were obtained in dark environment to preserve serum from light. In one aliquot, parameters were quantified within one week, through an automatic biochemical analyzer (Mindray BS-200, Alcyon, Italy) for the determination of serum concentration of ubiquitous intermediate metabolites and electrolytes (Na, K, Cl) and other elements (Mg, Zn and Cu). Reference intervals were adjusted for horse species and developed internally in the lab. Additionally, biochemical parameters obtained were compared to those reported by Kaneko and co-authors (2007) [21] and Latimer and co-authors (2011) [22].The other aliquot was used for the determination of α-tocopherol, by high pressure liquid chromatography coupled with an ultraviolet detector (HPLC-UV). All standards and solvents were purchased from Sigma Aldrich (Milan, Italy). Stock solution (1 mg/ml) of α-tocopherol was prepared in chloroform/methanol (50/50). For the calibration curve, standard stock solutions were diluted with methanol and kept

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frozen at -20°C, protected from light. Serum levels of α-tocopherol were measured at 280 nm.

Chromatographic separation was carried out on a Waters Symmetry C18 column (4.6 x 150 mm, particle size 5 μm, Waters, Milford, Massachusetts). The injection volume was 20 μl. The mobile phases used were acetonitrile/methanol/Milli-Q water (64.5/33/2.5) at 1 ml/min. Data were acquired and processed by Breeze Software (Waters, Milford, Massachusetts). Samples were prepared as follows: 0.3 ml of serum was vortexed with 0.6 ml of acetonitrile and centrifuged at 3500 g at 4°C for 10 min. The supernatant was dried under a stream of nitrogen and the residue was reconstituted in 0.15 ml of mobile phase [23, 24, 25].

Individual semen samples were collected through an artificial vagina (Colorado). After filtration, fresh gel-free ejaculate was checked for pH determination and inspected as colour. Subsequently, quantitative analysis considered sperm cell concentration and morphology, motility and integrity determined through the Microptic Sperm Class Analyzer® CASA System. Values were determined at 24 and 48 h at each scheduled sampling following the protocol described above.

2.6. Analysis of data and statistical methods

Values of investigated parameters were recorded for each horse, determined on T_0, T_1 and T_2 samples.

The statistical analysis of data was carried out by using a one-way ANOVA to assess differences of semen parameters in relation to the dietary treatment. Confidence intervals and grouping were adjusted according to Tukey method. All data were analyzed using SAS 9.2 (SAS Inst. Inc. Cary, NC). Statistical significance was set for p-value<0.05, whereas p-value<0.10 represented a trend.

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3. RESULTS

All animals enrolled in the trial appeared healthy throughout the experimental period.

TG stallions ingested 204±10.4mg/100 kg BW/d of all-rac-tocopheryl-acetate, representing 4-folds of daily intake of CG stallions. Daily intake of α-tocopherol in CG stallions appeared below recommendation for stallions at maintenance (NRC, 2007) [26].Alfa-tocopherol concentrations displayed to differ with marked significance ($p<0.0001$) between blood serum of TG and CG (3.22±0.19 *vs.* 1.01±0.04 μg/ml, respectively). Biochemistry of morning *vs.* evening blood samples highlighted differences in a non-statistical way and no circadian biorhythmic effects could be pointed out in blood serum concentrations in horses of this experimental trial.

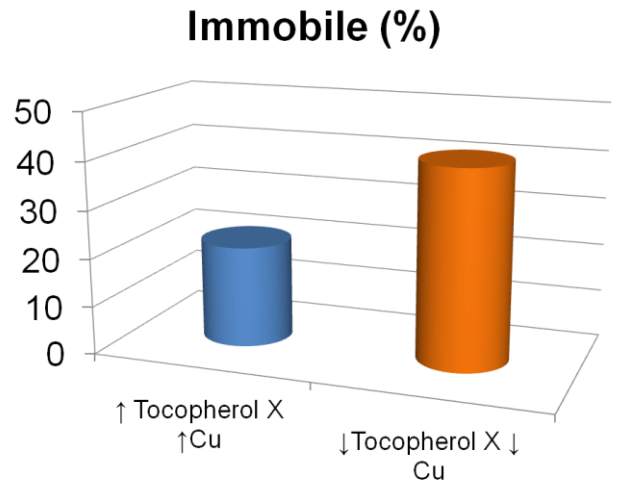
The metabolic profile of horses turned out to display concentrations of circulating metabolites falling within the physiological range for this species [21, 22]. With regard to semen quality, no statistic difference could be pointed out as to concentration, motile and progressive spermatozoa between the two groups (Table 2). Among parameters for the quality assessment of semen apparently not affected by nutritional management, the pH value may be a useful indicator for the assessment of the physiological condition (ranging between 7.2-7.7) because correlated with cell concentration (Mil/ml) in the horse [27]. The pH value in semen samples of both groups did not point to significant differences, ranging between 7.0 and 7.5. However, stallions in both groups have shown significantly higher percentages ($p<0.001$) of motile and progressive spermatozoa, in different proportions according to age (under 15 years: 77.9±4.2%; over 15 years: 54.9±2.2%) independently on tocopherol/Se supplementation. Interestingly, a significant ($p=0.039$) reduction of the percentage of immobile spermatozoa in stallion semen could be observed at the interaction of high circulating α-tocopherol and high copper levels if compared to low α-tocopherol x low copper levels (21.1±1.09% *vs.* 41.1±4.54%, respectively) (Graph 1). Circulating copper was negatively and weakly correlated ($\rho = -0.136$; $p=0.358$) with horse age in a non-significant way.

Results are resumed in Table 2.

[26] NRC. Nutrient Requirements of horses. 6 ed. Washington, D.C.: National Research Council, (2007).
[27] A.O. McKinnon, J.L.Voss. In: A.O. McKinnon, E.L. Squires, W.E. Vaala, D.Varner (eds) Equine reproduction. Williams & Wilkins, (1993).

Table 2. Intakes, serum concentrations of metabolites and semen parameters in horses enrolled 362 in the trial

	α-tocoferolo (µg/ml blood serum)			
Semen parameters	<2000	>2000	SEM	p
C.A.S.A.				
Sperm concentration (Mil/ml)	324	564	173	0.492
Immobile %	30.9	24.9	6.47	0.931
Straightness %	63.4	67.7	3.18	0.738



Graph 1. The histogram represents the percentage of immobile spermatozoa from horses as they result from the statistical interactions between circulating levels of α-tocopherol levels (modulated through the diet) and circulating levels of copper (higher in horses under 15 years of age) in horses involved in the trial.

4. DISCUSSION

The objective of this trial was to test the effect of dietary supplementation of α-tocopherol and Se in breeding stallions, to test the hypothesis on whether improvements of semen quality could be modulated by the diet. Note of worth, the higher circulating level of α-tocopherol was observed in the totality of supplemented stallions (TG) in comparison to that determined in horses from the CG. Alfa-tocopherol is a fat-soluble essential vitamin of vitamin E group characterized by lipophilic behaviour, of particular importance for stability and protection of cell membrane against ROS [28] at systemic level. The daily intake of α-tocopherol differed substantially between the two groups: TG horses ingested up to 4-folds

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the amount ingested by CG. However, blood serum circulating levels did not follow a proportional magnification of α-tocopehrol levels between horses of TG *vs.* CG. The non-supplemented stallions (CG) in this trial received a diet in which α-tocopherol was below NRC recommendations (2007) [26] and relative circulating levels in the bloodstream determined in the serum turned out to be deficient (< 1 μg/ml) [29, 30, 31]. Natural source of α-tocopherol is represented by fresh fodder whereas its content progressively diminishes in hay, due to thermo- and photolability of this fat soluble vitamin. In view of this fact, the introduction of α-tocopherol is of crucial importance in the diet of the stallion, commonly housed in individual box and fed on hay and concentrate feedstuffs as most. Against this background, systemic and local oxidative stresses are augmented during the stud season and semen production may be affected if dietary formulation does not meet nutritional and metabolic requirements. According to circulating levels of α-tocopherol (below or above 2 μg/ml) determined in horses involved in this experimental feeding trial, data on semen quality analyzed in the statistical model did not point to a significant difference on average values of parameters studied for the assessment of semen quality. This finding is in agreement with what reported by other authors [16]. However, other authors report contrasting results [12, 15, 16, 17]. In this trial, a systemic metabolic approach turned out to be useful to explain the effects of α-tocopherol/Se supplementation. A significant decrease of the percentage of immobile spermatozoa in the treated stallions was observed in this experiment as a result of the interaction of circulating α-tocopherol with Cu levels in the blood serum. Copper was not additionally supplemented in the diet of stallions and the circulating concentrations found in the bloodstream can be considered as the outcome of the metabolic status of each horse. The different extents of copper found in horses was negatively correlated with age, in a weak and non-significant way, but biological effects on the reduced percentage of immobile spermatozoa is significant. In fact, circulating Cu was higher in younger horses and this datum could be related to the bioavailability of Cu. On the basis of our results, the effect of the interaction between α-tocopherol and Cu on semen quality may find an explanation in the endogenous enzymatic systems to counteract ROS damage. Among such, SOD is a Zn/Cu-dependent system and Cu circulating levels may play a role in the synthesis for the SOD by spermatozoa, as well as in other cells of tissues of the animal body. Thus, α-tocopherol/Se supplementation in the diet of stallion may potentiate some metabolic activities in relation to the metabolic state and age of the horse and the role

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of circulating Cu could not be excluded. As recently reviewed by Finno and Valberg (2012) [13] and earlier studied by other authors [32], α-tocopherol possesses also modulating effects in terms of gene expression. Note of worth, the involvement of Cu in the effect of α-tocopherol/Se supplementation should be investigated further to understand the effective role in the antioxidant mechanisms applied directly on spermatozoa metabolic activity.

CONCLUSIONS

Dietary supplementation of α-tocopherol/Se in breeding stallions enrolled in this trial pointed to define an optimal level of blood serum concentration of tocopherol to support the metabolic needs for semen production of the horse. The supplementation did not produce a significant difference *per se* if not associated with a favourable availability of circulating Cu for antioxidant purposes, potentially involved in endogenous antioxidant systems. In conclusion, stallions up to 15 years old displayed favourable circulating Cu levels than older horses in this experiment and the dietary supplementation of α-tocopherol/Se turns out to potentiate the effects on semen quality, by inducing lower percentages of immobile spermatozoa.

CONFLICT OF INTEREST

Authors declare that no conflict of interest exists.

SOURCE OF FUNDING

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AUTHORSHIP

MGC conceived the study and drafted the manuscript; MGC, AT and IC carried out fieldwork activities; CD carried out statistical analyses; MGC, DG, FA and GB carried out lab analysis; MGC, DG, RC and WP interpreted results, supervised activities and revised the manuscript.

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Chapter 4

Baseline circulating levels of α-tocopherol in blood serum of feral Giara horses (Equus ferus caballus Linnaeus, 1758) and vitamin E status significantly vary with ALT from grazing to temporary captivity

Baseline circulating levels of α-tocopherol in blood serum of feral Giara horses (Equus ferus caballus Linnaeus, 1758) and vitamin E status significantly vary with ALT from grazing to temporary captivity

F. Accioni, W. Pinna, F. Pudda, P. Wolf, G. Boatto, M.G. Cappai.
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SUMMARY

Background: Alfa-tocopherol (α-TOH) is one isoform of fat soluble vitamin E group found in vegetation. Diets deficient in α-TOH may cause severe disorders in the horse. Large variations of circulating α-TOH in horse breeds can be expected.

Objectives: Giara horses may display breed-specific values of α-TOH in blood serum. Thus, baseline levels in a group of Giara horses before and after the temporary captivity in a wildlife rescue center were explored.

Study design: Baseline levels of α-TOH in the blood serum after grazing (in the wild, day 0) *vs.* hay based diet (captivity in the rescue center, day 28) with selected metabolites (ALT, Cholesterol, Triglycerides) were determined. From the hay-based diet the daily intake of α-TOH was calculated and used to interpret variations in circulating α- TOH and selected metabolites.

Methods: 6 adult Giara horses (body weight, 163 - 170 kg) were captured from the wild. On the same day (0d), blood samples were collected and processed for metabolic profile and α-TOH. In the wildlife rescue center, all animals received a same hay based diet (*ad libitum*) for 28 days. A second blood serum sample (28d) from the same individuals was processed for metabolic profile and α- TOH. HPLC-UV was used for α-TOH determination.

Results: Giara horses displayed α-TOH circulating levels below adequacy (2 μg/ml) for the horses on d0. Apparently, no clinical signs of deficiencies were found. Initial levels markedly ($p=0.020$) decreased after four weeks of captivity (-32.5%), when horses received a hay based diet. Circulating α-TOH and total cholesterol were significantly ($p=0.023$) and negatively ($\varrho=-0.648$) correlated. Significantly, ALT levels varied with vitamin E status.

Main limitations: Few animals due to wildlife conditions.

Conclusions: Circulating levels of α-TOH in the Giara horses enrolled may be useful to indicate the breed variance without apparent clinical vitamin E deficiency syndromes.

KEYWORDS: antioxidant; free grazing; feral horse; relict traits; vitamin E; wildlife rescue center

1. INTRODUCTION

Alfa-tocopherol (α-TOH) is one isoform of the fat soluble vitamin E group found in vegetation. Mammals are incapable of *de novo* synthesis thus α-TOH detected in the animal body comes from dietary sources. The biological value of α-TOH encompasses marked antioxidant activities [1],as it behaves as scavenger, potentiating the endogenous antioxidant systems that the animal employs to contrast lipid peroxidation. Limited to insufficient chronic supply of α-TOH in the diet appear to be associated with disorders of skeletal and myocardial muscles, as well as of the nervous system, though inconsistently reported in the literature [2, 3, 4, 5, 6, 7, 8, 9]. Neuroaxonal dystrophy is a disorder associated with low circulating value of α-TOH in the cerebrospinal fluid associated with lipid peroxidation [10].

In view of being essential for the animal, levels of circulating tocopherol are important for health maintenance. Recommendation of daily intake of α-TOH is 1-2 mg/ kg BW in a 500 kg BW of horse in light work [11].Unlike other fat soluble vitamins, α-TOH does not reach toxic levels, due to protection mechanisms through genetically encoded factors [12].Recently, α-TOH appeared to be involved in the metabolism of the hepatocyte with particular regard

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to the so called non alcoholic fatty liver disease. In such circumstances, circulating alanine aminotransferase (ALT) is used as indicator of the hepatocyte ballooning and liver damage 13. Adequate circulating values of α-TOH in the bloodstream of an average horse (body weight of 500 kg) is of 2 μl/ml, whereas values below 1.5 μl/ml are considered to be deficient [1]. However, breed variation in terms of circulating values of α-TOH in the horse are also reported [1, 13].

Feral Giara horses live in the wild on Sardinia isle, in the Mediterranean Sea. The peculiar haplotype set in the lineage highlights a conserved different origin from other domestic horse breeds reared in Italy [14, 15]. Giara horses are small sized (body weight range: 170-200 kg; wither's height: stallion, 125-130 cm; mare, 115-130 cm) [16, 17]. Such horses represent one of best genetically conserved breeds and display to possess relict traits and genetic variants that are almost totally lost in modern horse breeds [15]. Periodically, groups of feral Giara horses are gathered to monitor population size (Official registry of minor local breeds, Ministerial Decree 27/7/1990). All equines are also checked to test if positive to Equine Infectious Anemia (Coggins test).

It was hypothesized that free grazing Giara horses in the Mediterranean maquis might display α-TOH baseline levels capable to contribute to describe the breed variance of circulating values of this fat soluble vitamin E form, as described in the literature [1, 13].

This trial aimed to add acquaintances on α-TOH levels in blood serum of Giara horses and of selected metabolites considered to vary according to vitamin E status.

2. MATERIAL AND METHODS

All procedures reported in this trial belong to conventional practices for animal health assessment and wildlife monitoring; animal capture and blood sampling were carried out by expert veterinary personnel of the Autonomous Region of Sardinia. Animal handling complied with the recommendations of European Union Directive 2010/63/EU and

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following national guidelines concerning animal care. Biochemical profile and blood serum baseline levels of α-TOH in a group of Giara horses were determined. This study involved 6 Giara horses (4 mares and 2 stallions; estimated age: 3 to 4 years; body weight: 163 - 170 kg). All animals were captured from the wild by trained veterinary personnel, who read the electronic individual code number of each horse and collected individual blood sample for serological analyses. During the captivity period (horses were distributed in different paddocks, avoiding the presence of more than one stallion in each group), all animals received a same hay-based diet (*ad libitum*). Table 1 summarizes chemical and botanical composition of analyzed nutrients in the hay administered during the temporary captivity in the center.

Animals underwent the nutritional assessment, following the method described in Cappai et al. (2013) [18] modified here for the Giara horse. Briefly, body condition score (1 to 5-points scale), skeletal development and muscular condition (1 to 5-points scale) were evaluated.

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Table 1. Analyzed chemical composition of hay administered during the captivity period (4 weeks).

Analyzed nutrients	Hay
Dry Matter (g/kg as fed)	884.2
Crude Protein (g/kg DM)	116.6
Crude fat (g/kg DM)	36.8
α-tocopherol (g/kg DM)	13.6
Crude fiber (g/kg DM)	339.3
NDF (g/Kg DM)	699.1
ADF (g/Kg DM)	400.1
ADL (g/Kg DM)	53.1
Crude ash (g/Kg DM)	115.2

Two aliquots of blood serum samples (on day 0 and 28) from each horse were processed in the dark to prevent α-TOH degradation. Blood containing tubes were covered with tin-foils to protect blood from light. Samples were stored reporting individual label and date of sampling in polystyrene cases in the upright position in a refrigerated bag, to assure adequate temperature during the transfer to the laboratory.

All the laboratory procedures on whole blood were started within 6 hours from sample collection. Laboratory protocols for the collection, storage and analyses of blood samples were carried out in the dark, in order to avoid photo-degradation of α-TOH. Individual serum was screened for complete biochemical profile. Prior to chemical analysis of blood serum, individual blood samples were centrifuged at 1500 g at 4 °C for 10 minutes. An aliquot of each individual serum sample was stored in a sterile vial (2 ml) and frozen at -20°C, until further analyses. All the samples were analyzed within one week, through an automatic light-protected biochemical analyzer (Mindray BS-200, Shenzhen, China) for the determination of serum concentration of ubiquitous intermediate metabolites, enzymes, nutrients, macro- and micro-minerals. For the determination of α-tocopherol, high pressure liquid chromatography coupled with an ultraviolet detector (HPLC-UV) was carried out. All standards and solvents were purchased from Sigma Aldrich (Milan, Italy). Stock solution (1 mg/ml) of α-TOH was prepared in chloroform/methanol (50/50). For the calibration curve, standard stock solutions were diluted with methanol and kept frozen at -20°C, protected

from light. Serum levels of α-TOH were measured at 280 nm. Chromatographic separation was carried out on a Waters Symmetry C18 column (4.6 x 150 mm, particle size 5 µm, Waters, Milford, Massachusetts). The injection volume was 20 µl. The mobile phases used were acetonitrile/methanol/Milli-Q water (64.5/33/2.5) at 1 ml/min. Data were acquired and processed by Breeze Software (Waters, Milford, Massachusetts).

Samples were prepared as follows: 0.3 ml of serum was vortexed with 0.6 ml of acetonitrile and centrifuged at 3500 g at 4°C for 10 min. The supernatant was dried under a stream of nitrogen and the residue was reconstituted in 0.15 ml of mobile phase [19, 20, 21, 22].

On the same aliquot, 30 µl of serum from each individual were processed through electrophoresis to determine protein fractions on agarose gel, by means of a fully automated equipment (Pretty Interlab Srl., Rome). Electropherograms were scanned and interpreted through a dedicated soft ware (Elfolab, Interlab Srl., Rome). Serum protein fractions with particular regard to α_2 globulins where lipoproteins are accounted were analyzed in relation to vitamin E status. At this regard, the vitamin E status was determined by the ratio between circulating α-TOH and triglycerides plus total cholesterol.

Individual daily intake of feed was estimated on a 2% of BW, to compare recommendations of NRC on α-TOH daily intake (1 mg/kg BW) for a 500 kg BW horse at light work. Intakes were then calculated on average BW of the Giara horse. In addition, correlations between BCS and α-TOH levels as well as between α-TOH and selected circulating metabolites were carried out with Pearson's correlation analysis.

Data were analyzed by a general linear model (GLM) procedure of SAS 9.2 (SAS Inst. Inc. Cary, NC). The following model was used

$$yi,j = \mu + Di + Gj + Di * Gj + ei,j$$

where y is the dependent variable, µ is the overall mean, D and G are fixed factors (D, two levels: dietary regime grazing *vs.* captivity; G, two levels above and below 0.006) and *e* is the random error. Confidence intervals and grouping were adjusted according to Tukey method. All data were analyzed using Minitab software (Minitab Inc.). The statistic significance was set for p-value<0.05.

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3. RESULTS

3.1. Baseline values and animal response to captivity

All animals monitored before and after captivity appeared clinically healthy. The average BCS turned out to be of 2.79±0.19. On estimation of daily feed intake, α-TOH consumption was considered to range between 33.6 to 39.8 mg per horse during captivity. Baseline levels of α-TOH in blood serum significantly varied in horses before and after captivity (Δ[final-initial]α-TOH = -32.5%), highlighting the direct effect of variation of the diet on circulating values. Initial and final circulating levels of α-TOH in the blood serum of all horses were non significantly ($p=0.657$) and weakly ($\rho=0.248$) correlated with BCS. Circulating α-TOH and total cholesterol were significantly ($p=0.023$), negatively ($\rho=-0.648$) correlated. Table 2 summarizes results. Values of analyzed metabolites were found to be in the physiological range for horse species, except for α-tocopherol and alanine aminotransferase (ALT).

Table 2. Circulating values of α-TOH and other metabolites of interest in Giara horses detected at start (diet selected in the wild) and the end (after four weeks of hay feeding) of temporary captivity in the wildlife rescue center.

Animals (n.)	**Wild** **6**	**Captivity** **6**	**Pooled SD**	**p-value**
α-TOH (μg/ml)	0.43	0.29	0.07	0.020
Total Protein (g/l)	67.9	66.6	4.42	0.681
Albumins (g/l)	32.5	23.1	12.7	0.291
α_1-globulins (%)	3.77	7.26	6.02	0.405
α_2-globulins (%)	9.57	15.2	6.87	0.216
β-globulins (%)	12.7	13.8	11.6	0.895
Total Triglycerides (mg/dl)	27.7	20.3	6.93	0.144
Total Cholesterol (mg/dl)	48.0	60.1	8.41	0.056
Alanine aminotransferase (U/l)	68.1	28.1	23.9	0.032

4. DISCUSSION

This trial was conceived to contribute to shed a new light on the breed variance of circulating α-tocopherol, by comparing blood serum levels considered to be adequate for the domestic modern horse (2 μg/l of serum) with those detected in the blood serum of free grazing Giara horses. The rationale behind this trial resides on different key principles of animal nutrition, health and natural diet, in view of the interplay between animals and environment through selection for maintenance of antioxidant status. The biological activity of α-tocopherol, as an

important natural isoform of fat soluble vitamin E group, is of particular interest because of its essential role for the animal (incapable of *de novo* synthesis). Moreover, unlike other fat soluble vitamins (namely vitamin A or D, for instance) it circulates in the bloodstream in its native form transported by LDL and HDL [1] and does not need to be metabolized to be activated in the animal body. Thus, α-TOH may represent a marker of feed selection and nutrient intake, being the most biologically active compound with antioxidant properties. It is well known that tocopherols are most abundant in leaves whereas tocotrienols are in seeds [13]. Thus, the determination of circulating α-TOH may reflect the selection in the natural diet of leafy green sources. Though tocopherols can be found in plant seeds too, γ-tocopherol is abundantly synthesized while α-TOH only to a lesser extent. Both carotenoids and tocopherols are reported to be the most abundant groups of lipid-soluble antioxidants in chloroplasts of plants. To such an extent, α-TOH content in plant species dramatically drops after drying to hay, commonly administered to ranged animals. Hay contains much lower amounts of α-TOH than in same plant species when green. Free grazing Giara horses in the wild Mediterranean maquis display peculiar circulating baseline levels of α-TOH despite grazing free in nature. No signs among those described to involve horses fed with vitamin E deficient diets could be pointed out to affect animals enrolled in this trial. As expected, initial values of circulating α-TOH markedly decreased when horses were fed a hay based diet after four weeks of captivity. Though the effect of the diet on circulating α-TOH levels in the bloodstream of the horse is established, it is also known that the variations due to age, sex, breed and physiological state of the animal may also have an impact on blood serum values [12]. The determination of α-TOH levels in the bloodstream of Giara horses covers several aspects both of nutritional and health importance. Baseline values of circulating α-TOH detected in the Giara horses until free grazing in the Mediterranean maquis appeared to be low if compared to those identified to be adequate (>2 μg/ml), marginal (between 1.5 and 2 μg/ml) and deficient (below 1.5 μg/ml) [1] in the domestic modern horse. Such categories were developed on the basis of potential disorders correlated with dietary chronic deficiency of vitamin E in the stabled horse, according to which dietary supplementation may be planned [10]. In this trial, all Giara horses appeared healthy and no signs indicating disorders from Vitamin E deficiency could be pointed out, despite α-TOH circulating values could be considered as deficient. In fact, baseline levels of circulating α-TOH detected in Giara horses when captured from the wild displayed to be below those associated with deficiency in the domestic horse, showing a relative concentration of -78.5% lower. Whether this finding

depends on low requirements in this old horse breed strictly adapted to environment is still under investigation.

During the month of captivity, α-TOH in the bloodstream dramatically decreased to about one third of levels found at start. Indeed, the hay based diet did not supply the horse with adequate amounts of α-tocopherol. It is known that α-TOH content in fresh forage diminishes progressively after the cut and throughout the process of drying to hay. In fact, the daily supply of α-TOH (13.6 mg α-tocopherol/kg DM in hay) has meant a maximum estimated daily intake of 40.7 mg of α-TOH for a horse of 170 kg of live weight. If compared to NRC recommendations of α-TOH daily intake for the horse (2007) [11] ranging between 1 and 2 g of α-TOH / 500 kg BW horse in light work, the best prediction of α-TOH requirement for the Giara horse should be estimated according to the metabolic weight, for which the recommended α-TOH supply is 5 mg/kg BW0.75/d. This translates into 235 mg/ 170 kg BW Giara horse/d. Thus, the hay based diet turned out to supply α-TOH below NRC (2007) recommendations [11] and, in any case, circulating levels of α-TOH determined at start and at the end of the captivity period should be both considered as deficient. The dietary supply below requirements probably led to α-TOH depletion from body stores (if any?), emphasized by the circulating levels found at the end of the captivity period [Δ(final – start) = -32.5%]. Indeed, the rapid drop of circulating values seems to translate into the depletion of potential body stores, though levels of circulating α-TOH at start already appeared to be deficient [1]. Against this background, it could be postulated that Giara horses can adapt successfully to lower circulating levels of α-TOH without developing clinical signs. Whether this finding is driven by feed selection or due to different efficiency of digestion and absorption is still unknown. The BCS of horses enrolled appeared to be on average associated with normal body weight for the breed and no significant correlations between fatness and circulating α-TOH were pointed out. Worth of note, total cholesterol levels in the bloodstream appeared significantly and negatively correlated with circulating α-tocopherol. The literature reports the effect of α-TOH supplementation on the reduction of circulating HDL in human patients under therapy with statins [23]but not of LDL, thus decreasing the total cholesterol levels. In the same study, effects from supplementation of α-TOH were no more evident during the wash out period of two weeks, and this may be in agreement with our findings. In fact, comparatively, at decreased levels of α-TOH in the bloodstream, total cholesterol increased. In addition, at decreased levels of α-TOH in the

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bloodstream total triglyceride decreased. The relationship between α-tocopherol, total cholesterol and total triglycerides in the bloodstream are known to be correlated [24, 25] and ratios between such metabolites are used to estimate the vitamin E status in man. The calculated α-tocopherol: total cholesterol + total triglycerides to assess vitamin E status of Giara horses before and after the captivity period turned out to be 0.0006 and 0.0003, respectively. Thus, the vitamin E status of Giara horses decreased to one half after one month of hay based diet. The variation of alanine aminotransferase (ALT) as sign of hepatic damage, is markedly and significantly ($p=0.016$) varying in relation to vitamin E status, and such significance is higher than the variation of ALT when the diet effect is considered ($p=0.032$).

If health conditions are considered in view of the circulating α-TOH levels found in the Giara horse, then α-TOH rich diets based on free grazing in the natural Mediterranean maquis may point to different utilization of such compound in Giara horses from that in domestic modern horse breeds. Selection of particular traits in sport horses may have led to α-TOH increased requirements. Storage appears not to be a pivotal need for the maintenance of cell membrane stability of muscle masses for Giara horses, like instead potentially required by athlete horses undergoing to training. However, circulating levels of triglycerides and total cholesterol responded to blood serum concentration of α-TOH as expected, still within the physiological range for this species.

Findings reported in this trial pave the way to further investigations on breed specific levels of circulating α-TOH in the blood serum of free grazing horses and more in general on breed-specific levels found in equines [26],highlighting the potential effects of genetically encoded factors of α-TOH metabolism.

5. CONCLUSIONS

Circulating values of α-TOH in the blood serum of Giara horses displayed to be deficient despite the potential large intakes on a daily basis from grazing in spontaneous vegetation. In fact, values found in Giara horses appeared to be very low if compared to α-TOH circulating values considered as adequate in modern horse breeds. The vitamin E status dramatically

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decreased after four weeks of hay-based feeding during the temporary captivity in the totality of animals, pointing to body store depletion. The vitamin E status emphasized also the variation of blood serum levels of ALT in a very marked way than the sole variation following the feeding regime. No clinical signs from such deficient vitamin E status were pointed out, though ALT levels were found above the upper values of the reference range for the horse both at start and end of the captive period. It is necessary to underline that other antioxidant compounds than α-TOH can compose the diet of the grazing horse, thus minimizing the potential damage from lipid peroxidation. However, whether circulating values found in this trial should be interpreted in the light of different nutritional needs due to genetic relict traits of Giara horses, is difficult to state. Arguably, the mutual interplay between grazing animals and spontaneous vegetation may have led to the natural adaptation of feral Giara horses to feeding sources and relative seasonal nutrient and energy availability in nature, from coping ability across centuries.

Ethical Consideration: This trial was approved by the Ethic Committee OPBSA of the University of Sassari, no. 00003185.

Competing interests: Authors declare that no competing interest exists.

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Part B

SUPRAS-based matrix-independent platforms for quantifying multi compounds in biological matrices by LC-MS/MS for forensic, clinical and food quality control purposes

Chapter 5

SUPRAS extraction approach for matrix-independent determination of amphetamine-type stimulants by LC-MS/MS

SUPRAS extraction approach for matrix-independent determination of amphetamine-type stimulants by LC-MS/MS

F. Accioni, D. García-Gómez , E. Girela, S. Rubio

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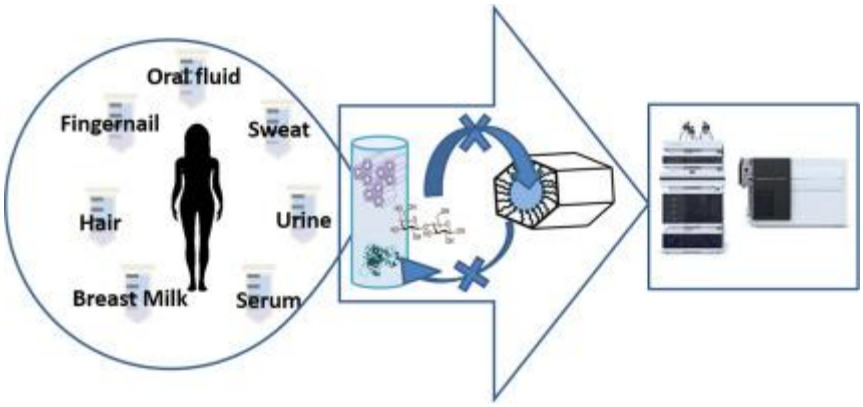

ABSTRACT

Monitoring of amphetamine-type stimulant (ATS) confronts clinical labs with a high number of samples involving a variety of biological matrices. Liquid chromatography-tandem mass spectrometry (LC-MS/MS), routinely used for confirmation of ATS abuse, requires of laborious and matrix-dependent sample treatment methods, this increasing analysis time and cost. In this work, a universal and single-step sample treatment, based on supramolecular solvents (SUPRAS), was proposed for simplifying ATS confirmation in seven biological matrices. The SUPRAS was synthesized *in situ* in the sample (900 μL of basified oral fluid, urine, serum, sweat or breast milk or 50 mg of digested hair or fingernails) by the addition of hexanol (200 μL) and tetrahydrofuran (900 μL). The mixture was vortex-shaken and centrifuged and the SUPRAS extract was subsequently analyzed by positive ion mode electrospray LC-MS/MS. The method was fully validated for amphetamine (AMP), methamphetamine (MA), 3,4-methylenedioxyamphetamine (MDA), N-ethyl-3,4-methylenedioxyamphetamine (MDEA) and N-methyl-3,4-methylenedioxyamphetamine (MDMA). Maximum ion suppression or enhancement was 9% and 7%, respectively, and extraction recoveries (87-111%) and within- (0.1-6.7%) and between-day (0.3-9.7%) CVs were all within required values. The lower limits of quantification (LLOQ) for biological fluids (5 ng/mL), and hair and fingernails (100 ng/g) were all well below the cut-offs established by worldwide organizations. Confirmation of MDA was carried out in five urine samples that tested positive for ATS by immunoassay. The SUPRAS-LC-MS/MS

methodology succeeded in developing a hitherto unexplored and universal tool for quantifying ATS in a comprehensive pool of biological matrices of interest in forensic and clinical samples.

KEYWORDS: amphetamines; biological samples; sample treatment; supramolecular solvents; liquid chromatography; tandem mass spectrometry

1. INTRODUCTION

The need for new methodologies that allow the determination of amphetamine and their derivatives (MA, MDA, MDEA and MDMA) in biological matrices is supported by the alarming data provided by different worldwide reports [1]. The driving force causing expanding abuse of amphetamine-type stimulants (ATS) is their pharmacological activity on the central nervous system, linked with increasing of energy, endurance and sociability [2]. Around 2.1 million young adults used MDMA – "ecstasy"- worldwide during 2015 while for AMP and MA the numbers were around 1.3 million [3]. World Drug Report 2016 shows a rising trend in consumption being ATS the second most consumed illicit drugs [4].

ATS, similarly to other illicit drugs, are determined in a variety of biological matrices with very different purposes, including workplace testing, Driving Under the Influence of Drugs (DUID) programs, drug consumer follow-up, gestational or newborn exposure, post-mortem toxicology, drug facilitated sexual assaults, and so on [5]. Matrix selection depends on the purpose of the analysis as well as on the advantages and limitations that each matrix brings out [6, 7]. Thus, it is important to consider the required detection time window, which may range from hours to a few days for biological fluids and from months to years for hair and fingernails [8]. This broader detection window has permitted the use of hair as an

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attractive matrix to assess gestational exposure [9]. On the other hand, sample collection convenience is essential for on-site drug testing. Sampling of oral fluid is preferred for workplace, antidoping testing and DUID programs because is non-invasive and less subject to adulteration than urine and, on the contrary than blood, it does not require specialized staff [10, 11, 12]. Correlation between drug concentration and pharmacodynamic effects is required for judicial settings and, in this sense, oral fluid and blood show better correlation with impairment performance that urine [13]. Breast milk is interesting for assessing newborn exposure [14] while sweat patches provide a qualitative record of drug consumption over the period of observation [15]. In short, clinical labs are routinely confronted with the analysis of a huge number of samples involving many different biological matrices.

ATS screening is mainly based on class specific immunoassays but the lack of specific drug identification and cross-reactivity with unrelated medications demands for drug confirmation and quantitation by a more selective analytical technique [16]. Traditionally, gas chromatography-mass spectrometry (GC-MS) has been used for ATS determination; however, the need for ATS derivatization has fostered the use of LC-MS/MS [7]. A critical point with this technique is its susceptibility to matrix effects, which often compromises sensitivity and selectivity and consequently the accuracy of its application [17, 18]. As a result, sample treatment, which is matrix-dependent, often involves extensive, time-consuming, non-green, and unspecific procedures and consequently, mostly of the reported methods have been only validated for single biological matrices [19, 20, 21, 22, 23, 24, 25,

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26]. Therefore, the development of a unique, simple, and fast sample treatment, integrating both ATS extraction and cleaning-up of matrix interferences, and applicable to the major types of biological matrices of interest for the control of ATS abuse by LC-MS/MS, would be of interest for clinical and toxicological labs. In this work, we try to succeed this aim with the use of supramolecular solvents (SUPRAS).

SUPRAS are nanostructured liquids generated from colloidal solutions of amphiphiles by spontaneous processes of self-assembly and coacervation [27]. They are highly ordered systems showing well-differentiated regions. An outstanding feature is that their structure, composition and properties can be tailored at will by selecting the environmental conditions for amphiphile aggregation. In this way, water-induced SUPRAS with restricted-access properties (SUPRAS-RAM) have been synthesized from colloidal solutions of alkanols in tetrahydrofuran [28, 29] giving solvents made up of inverted hexagonal aggregates, with the hydrophilic alcohol heads surrounding aqueous cavities and the lipophilic chains dissolved in tetrahydrofuran (Figure 1). These different polarity regions imply that SUPRASs can interact in several ways with low-molecular weight solutes, whilst polysaccharides and proteins are excluded by size and precipitation, respectively [28]. Furthermore, the size of the aqueous cavity can be tuned controlling the initial conditions and, because of the non-covalent nature of their internal bindings, the tailor-made synthesis is completely reversible [28, 29]. SUPRAS-RAM have been used in food and environmental analysis because of their high capacity to clean up complex matrices, rich in interferences, and to extract the target compounds with optimum recoveries [30, 31, 32]. These properties have been successfully proved for a wide range of chemicals, from very low (e.g. vitamin E) to high (e.g. hydrazine) polarity, demonstrating in this way that SUPRAS composition and nature can be tailored to match the target analytes [27]. In view of all these facts, it is the aim of this work to develop and validate a universal sample treatment platform, based on SUPRAS-RAM, which may

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allow the determination of ATS by LC-MS/MS in human biological matrices of toxicological and forensic interest.

2. MATERIAL AND METHODS

2.1. Chemicals and reagents

ATS standards (AMP, MA, MDA, MDEA and MDMA), the internal standard (IS) methamphetamine-D14 (MA-D14), methanol, acetonitrile, ammonia (25%), sodium hydroxide and formic acid were supplied by Sigma-Aldrich. The reference materials (RM) Medidrug® DOA-I S low and Medidrug® WDT Confirm U -25% were obtained from LGC Ltd. The first RM is a lyophilized serum that contains 28 drugs, including the target ATS at 25 μg/L, while the second RM is a lyophilized urine that contains 55 drugs including ATS (150 μg/L each) at concentrations that are -25% of the recommended cut-off by the European Workplace Drug Testing Society (EWDTS). The RM DHF 2/12-A HA was purchased from ACQ Science. This RM is a powdered hair that contains 16 illegal drugs including ATS at the following concentrations: AMP (1170 ng/g), MA (797 ng/g), MDA (428 ng/g), MDEA (589 ng/g) and MDMA (1740 ng/g). Tetrahydrofuran was obtained from Panreac and 1-hexanol from Merck. All solvents were LC-MS grade. Type I water was obtained from a purification system (Millipore).

2.2. Solutions

Stock solutions for ATS standards and the internal standard MA-D14 (25 μg/mL each) were prepared in methanol and stored at -20 °C. These solutions were proved to be stable for at least 2 years. Working solutions were made daily by proper dilution of the stock solutions with water. Their stability in the whole linear calibration range under the working experimental conditions (autosampler at 20 ºC) was proved to be at least 3 days.

2.3. Sample collection and pretreatment

Human blank biological samples were collected from volunteers in agreement with the "Ethics Committee of Andalusian's Biomedical Research", and the Declaration of Helsinki. Spot urine samples were collected only during early morning. For saliva sampling, participants were asked not to drink, eat, smoke or chew gum one hour before collection, which was carried out by spitting in a glass test tube. Serum samples were obtained by centrifugation of blood samples in EDTA-containing tubes and transferred into polypropylene tubes. Breast milk samples were donated by 27 to 39-year-old mothers in her

first month of breastfeeding. Hair was sampled following the guidelines set by the Society of Hair Testing [33], decontaminated following the Imbert et al. method [34], and subjected to milling until a particle size below 50 μm was obtained. Fingernails clipping and cleaning were conducted as proposed by Lin et al. [8]. Sweat was sampled by using sterile patches (3M Medical Sciences) attached to volunteers' forehead, upper chest and back for 1.5 hours during physical activity. An additional set of five anonymized urine samples obtained from individuals following a rehabilitation program, which were positive to the Ecstasy assay by immunoassay (Dimension, Siemens), was supplied by the Toxicology Service of the University Campus Hospital of Granada (Spain) in accordance with the Ethics Committee of Andalusian's Biomedical Research (ECABR) approval. All samples thus obtained were immediately analyzed and/or stored at -20°C for further analysis.

2.4. SUPRAS-based extraction

Taking advantage of the high proportion of water in several of the selected biological fluids (i.e. saliva, serum, urine, breast milk and sweat), a water-induced SUPRAS-RAM was in situ produced in the sample. For this purpose, 900 μL of biological fluid basified with NH3 25% (0.1 M) were mixed with 900 μL of tetrahydrofuran and 200 μL of 1-hexanol in a 2-mL Eppendorf tube. The SUPRAS spontaneously and instantaneously was formed in the bulk solution by self-assembly and coacervation. The mixture was vortex-shaken in a Heidolph Reax vortex mixer for 10 minutes and centrifuged in a MPW -350R centrifuge (MPW Med-Instruments) at 21125g for 5 minutes. The SUPRAS, less dense than water, separated from the bulk solution as an upper layer. In the case of solid biological matrices, i.e. hair and fingernails, the samples were previously subjected to alkaline digestion (1 mL of 1M NaOH per 50 mg of sample) at 80 ºC for 1 h according to reported procedures8 and then, the resulting solution was subjected to the procedure stated above. All SUPRAS extracts were fortified with MA-D14 (25 μg/L) before analysis. The stability of ATS in these extracts at room temperature and 4ºC was at least 3 days and 2 weeks, respectively.

2.5. LC-MS/MS assay

LC-MS/MS assays were run in an Agilent Technologies 1200 series LC coupled to a 6420 triple quadrupole mass spectrometer with an electrospray ionization source (ESI).

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Chromatographic separation was carried out onto a Kromasil C18 column (4.6 X 150 mm i.d., particle size 5 μm from Análisis Vínicos, Spain). The mobile phase was made up of a 0.1% formic acid aqueous solution (solvent A) and a 0.1% formic acid methanolic solution (solvent B). The elution gradient was as follows: 20% of B until 7.50 minutes, from 20% to 70% of B at 10 minutes, and 20% of B from 10.50 to 15 minutes. The injection volume was set at 2 μL. Tandem mass spectrometry parameters were optimized by direct infusion. The most abundant fragments for each ATS and the internal standard MA-D14 were used as quantifier and qualifier (see operating settings in Table 1). ATS were quantified based on calibration curves constructed from solutions containing standards in water in the range 5-250 ng/mL (25 ng/mL of MA-D14), by measuring peak-area ratios (ATS standard vs MA-D14). Figure 2A shows the extracted ion chromatograms (EICs) obtained for aqueous standard solutions of ATS, at 25 ng/mL each, monitored at the respective quantification transitions (Table 1).

Table 1. Mass spectrometry parameters for ATS quantification[a]

ATS	Quantification transition, m/z (collision energy in volts)	Confirmation transition, m/z (collision energy in volts)
AMP	136→91 (18)	136→65 (40)
MA	150→91 (18)	150→65 (40)
MDA	180→163 (10)	180→105 (10)
MDEA	208→163 (22)	208→105 (10)
MDMA	194→163 (26)	194→105 (10)
MA-D14	164→130 (10)	164→98 (22)

[a]Source settings: source gas: 50 psi of N_2 at 350 °C; capillary voltage: +3500 V; and fragmentor voltage: 95 V.

2.6. Method validation

Calibration curves were run by spiking ten blank samples of each of the seven biological matrices under study with a mixture of ATS (0, 5, 10, 25, 50, 100, 250, 500 ng/mL for oral fluid, serum, urine, sweat and breast milk and 0, 100, 200, 500, 1000, 2000, 5000, 10000 ng/g for hair and fingernails) and the internal standard MA-D14 (25 ng/mL for biological fluids and 1000 ng/g for solid matrices). Each calibration standard was analyzed run in the LC-MS/MS system in triplicate. Calibration curves were constructed by plotting peak-area ratios (ATS standard vs MA-D14) as a function of the standard concentration. Least-square regression was used to describe the concentration-response relationship.

Matrix effects (ME) were assessed by the method proposed by Matuszewski et al. [35]. For this purpose, calibration curves (n=5) were run from standards of ATS in the range 5-250 ng/mL, at seven different concentrations, and the corresponding slopes calculated by least-square regression. The mean value of these slopes was compared to those obtained from the analysis of ten different lots (i.e. from ten different subjects) of each type of biological matrix, fortified with ATS after extraction with the SUPRAS-RAM. The IS MA-D14 was also added to both standards and SUPRAS extracts. Matrix effects, expressed as percentage, were calculated by dividing the mean values of sample slopes by the mean value of the neat standard slopes.

Precision was evaluated by spiking pooled (n=5) blank samples with three ATS concentrations (5, 25 and 250 ng/mL for biological fluids and 100, 1000 and 10000 ng/g for hair and fingernails) and the IS MA-D14 (25 μg/L for biological fluids and 1000 ng/g for solid matrices). Seven aliquots of these samples were daily analyzed and the experiment was repeated on four different days by running the same samples.

Accuracy was evaluated by spiking blank samples (n=10) with ATS and MA-D14 at the same concentrations than those used for the assessment of precision. The accuracy for diluted urine was also evaluated by spiking the samples with 5000 and 7500 ng/mL and diluting them with water by factors of 100 and 50, respectively. Concentrations of ATS in the spiked samples were calculated from calibration with standards in water and the accuracy was reported as percent of the nominal value. Likewise, three reference materials for ATS in urine (Medidrug® WDT Confirm U -25%), serum (Medidrug® DOA-I S low) and hair

[35] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem., 75 (2003) 3019-3030.

(DHF 2/12-A HA), containing many other drugs illicit drugs, were used for assessment of the accuracy of the method.

3. RESULTS AND DISCUSSION

3.1. ATS SUPRAS-based extraction

A good knowledge of the physical-chemical characteristics of both analytes and extractant is essential to develop efficient extraction processes. ATS are basic (pKa values in the range 9.5-9.9) and polar (log Kow in the range 1.67-2.34), and contain aromatic rings and hydrogen bond donors/acceptors in their structure. The SUPRAS selected for extraction of ATS was made up of inverted hexagonal aggregates of hexanol, with the polar groups surrounding water cavities and the hydrocarbon chains dispersed in THF (Fig. 1). In this way, ATS can be solubilized in the SUPRAS by mixed-mode mechanisms, that is, polar and hydrogen bond donor/acceptor interactions with alcohol groups and dispersion interactions with the hydrocarbon chains.

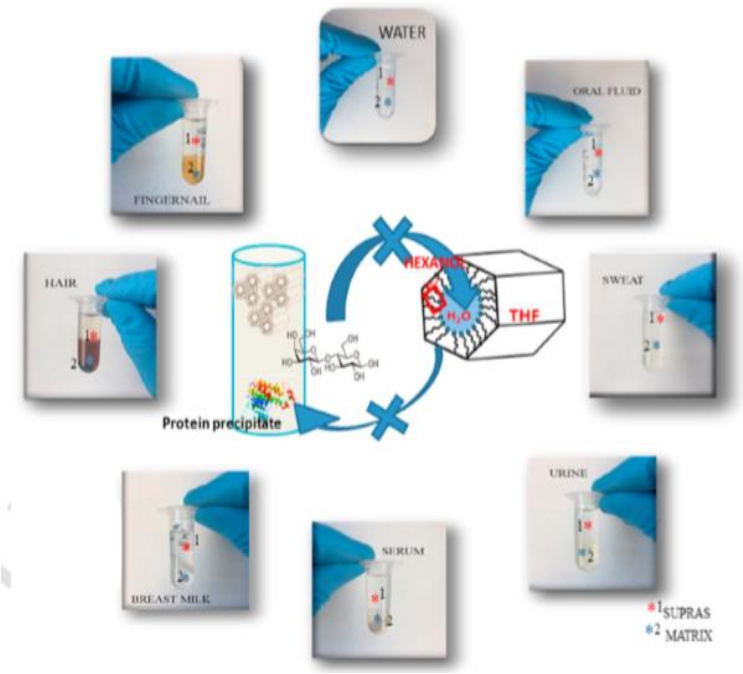

Figure 1. Photographs corresponding to the extraction of ATS from a fortified aqueous solution and seven biological matrices with a hexanol-based SUPRAS-RAM and schematic showing the structure of the SUPRAS and the mechanisms for proteins and carbohydrates removal.

The ability of hexanol-based SUPRAS to extract ATS was preliminary investigated using fortified aqueous solutions since biological fluids and digested hair and fingernails consist

essentially of water. For this purpose, SUPRAS of different composition and vacuole sizes were in situ produced in the aqueous solution by keeping constant the proportion of hexanol (10%) and varying the relative proportion of water and THF [29]. The pH of the aqueous solution was adjusted to study the behavior of both charged (pH = 5) and neutral (0.1 M NH3) ATS. The synthesis of SUPRAS was carried out following the procedure specified in Materials and methods.

Table 2 shows the composition of the synthetic solutions, as well as the respective percentages of water in the SUPRAS (calculated with a Karl Fischer coulometric titrator from Metrohm) and the volumes of SUPRAS produced. The proportion of water in the SUPRAS increased as the percentage of THF in the synthetic solution did, that increasing the volume of SUPRAS produced. Recoveries obtained for ATS were highly dependent on both the composition of the SUPRAS and the chemical form of drugs (Table 2). SUPRAS containing high percent of water were able to extract the neutral form of ATS from basic aqueous solutions efficiently (recoveries were in the range 89-102%). So, the SUPRAS selected as extractant was that synthesized by adding hexanol and THF to basified samples (0.1M NH3) at the proportions 10%, 45% and 45%, respectively.

Table 2. Experimental conditions for SUPRAS formation, percentage of water in the SUPRAS, volume of solvent produced and recoveries obtained for ATS

[a] Composition of the synthetic solution			Water content in the SUPRAS (%)	Volume of SUPRAS produced (µL)	[b]ATS recoveries (%)
Hexanol (%)	THF (%)	Water (%)			
10	5	85	1.3	252	-
10	15	75	5.2	351	-
10	25	65	7.4	488	8-14
10	35	55	9.7	679	8-25
10	45	45	27.1	945	8-34
10	25	[c]65	7.4	488	74-80
10	45	[c]45	27.1	945	89-102

[a] Total volume of the synthetic solution: 2 mL
[b] Concentration of each ATS in the aqueous solution: 100 ng/mL
[c] Aqueous 0.1 M NH_3

Additionally, these SUPRAS have the ability to behave as restricted access liquids through chemical and physical mechanisms [**Error! Bookmark not defined.**]. Thus, proteins precipitate or flocculate by a combined effect of the decrease of the dielectric constant in the presence of THF and the formation of complexes with the amphiphile. On the other hand, the high polar carbohydrates are not incorporated to the aqueous cavities of the hexagonal aggregates by size exclusion.

Figure 1 shows photographs of the extraction procedure of ATS from the different biological fluids and digested hair and fingernails using the selected SUPRAS-RAM. A large precipitate or flocculate of proteins was well observed for some biological samples such as breast milk, serum and hair, while it was quite low for urine and fingernails. No precipitate was found for oral fluid and sweat matrices. It is worth noting that, independent of the biological matrix, colorless SUPRAS extracts were always obtained.

On the basis of these preliminary results, a general method for the quantification of ATS in different biological matrices, based on the combination of SUPRAS-RAM and LC-MS/MS, was developed and fully validated following standard guidelines [36, 37,].

[36] US Food and Drug Administration (FDA). Guidance for Industry: Bioanalytical Method Evaluation, 2017 https://www.fda.gov/downloads/Drugs/Guidance/ucm070107.pdf. (Accessed 10 December 2017).

3.2. Method Validation

3.2.1. Calibration model

Calibration curves for ATS in biological samples were linear in the concentration ranges: 5-500 ng/mL for biological fluids and 100-10.000 ng/g for hair and fingernails. Table 3 lists the calibration curve parameters (i.e. slopes, y-intercepts, determination coefficients and standard deviations of residuals) for each ATS and sample as calculated by least-square regression. In the case of hair and fingernails, calibration curve parameters were calculated from the concentration of ATS in the digested samples in order to compare the results with those obtained from biological fluids. Slopes for each ATS was quite independent of the type of biological sample, that indicating the potential of the SUPRAS-RAM to provide a matrix-independent treatment method. The back-calculated concentrations for all calibration samples were within ± 15% of the nominal concentrations, which was in good agreement with established criteria [36, 37]. The lower limit of quantification (LLOQ) for the target ATS, defined as the lowest concentration of the calibration curve that can be measured with a coefficient of both variation and accuracy less than 20%, was 5 ng/mL for biological fluids and 100 ng/g for hair and fingernails. Both LLOQ values were below the most restrictive cut-offs proposed by the Substance Abuse and Mental Health Services Administration (SAMHSA) as confirmatory levels by LC-MS/MS, namely 25 ng/mL and 200 ng/g [16]. The extracted ion chromatograms obtained from the analysis of blank biological fluids, hair and fingernails, fortified with ATS at the LLOQ level, are shown in Figure 2 BC-H.

[37] European Medicines Agency (EMA). Guideline on bioanalytical method validation, 2011 http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf ., (Accessed 10 December 2017).

Table 3. Calibration curves parameters for ATS in different biological matrices

ATS		Oral fluid	Sweat	Urine	Serum	Breast milk	Hair	Fingernails
AMP	[a](Slope ± SD)x10^{-2}	1,83±0,01	1,80±0,01	1,80±0,01	1,93±0,01	1,97±0,01	1,90±0,01	1,87±0,01
	y-Intercept ± SD	-0,01±0,02	0,01±0,02	0,01±0,05	-0,01±0,08	-0,06±0,04	-0,03±0,01	-0,04±0,01
	[b]R^2	0,994	0,999	0,990	0,999	0,999	0,993	0,996
	[c]Sy.x	0,32	0,04	0,17	0,16	0,02	0,06	0,05
MA	[a](Slope ± SD)x10^{-2}	0,83±0,04	0,80±0,04	0,80±0,04	0,86±0,04	0,82±0,06	0,82±0,04	0,82±0,06
	y-Intercept ± SD	0,01±0,01	0,01±0,01	0,01±0,03	-0,01±0,01	-0,06±0,03	0,01±0,01	-0,03±0,01
	[b]R^2	0,996	0,999	0,991	0,999	0,996	0,993	0,993
	[c]Sy.x	0,04	0,02	0,02	0,02	0,05	0,01	0,01
MDA	[a](Slope ± SD)x10^{-2}	2,45±0,01	2,78±0,02	2,45±0,01	2,77±0,01	2,67±0,01	2,77±0,02	2,72±0,02
	y-Intercept ± SD	0,08±0,07	-0,01±0,01	0,01±0,09	-0,01±0,05	0,02±0,05	0,01±0,04	-0,02±0,06
	[b]R^2	0,993	0,999	0,999	0,999	0,998	0,997	0,996
	[c]Sy.x	0,03	0,02	0,04	0,06	0,06	0,06	0,07
MDEA	[a](Slope ± SD)x10^{-2}	6,65±0,01	7,13±0,01	6,70±0,01	7,33±0,01	7,04±0,01	6,79±0,01	6,75±0,01
	y-Intercept ± SD	0,05±0,06	-0,01±0,09	-0,06±0,04	-0,04±0,04	0,05±0,05	0,04±0,05	0,03±0,05
	[b]R^2	0,995	0,999	0,997	0,999	0,999	0,995	0,998
	[c]Sy.x	0,08	0,02	0,04	0,05	0,02	0,09	0,04
MDMA	[a](Slope ± SD)x10^{-2}	3,04±0,01	2,99±0,01	2,77±0,01	2,95±0,01	2,71±0,01	2,76±0,01	2,71±0,01
	y-Intercept ± SD	-0,01±0,01	0,01±0,04	-0,01±0,03	0,01±0,02	-0,01±0,03	0,01±0,03	0,01±0,04
	[b]R^2	0,997	0,999	0,987	0,999	0,999	0,997	0,999
	[c]Sy.x	0,02	0,02	0,08	0,02	0,01	0,02	0,01

[a] Units are ATS/IS peak area ratio per ng/mL ATS. Number of calibration curves = 10. Slopes for hair and fingernails were calculated from ATS concentrations in the digested samples (see section SUPRAS-BASED EXTRACTION in Materials and Methods).
[b] Coefficient of determination
[c] Standard deviation of the residuals

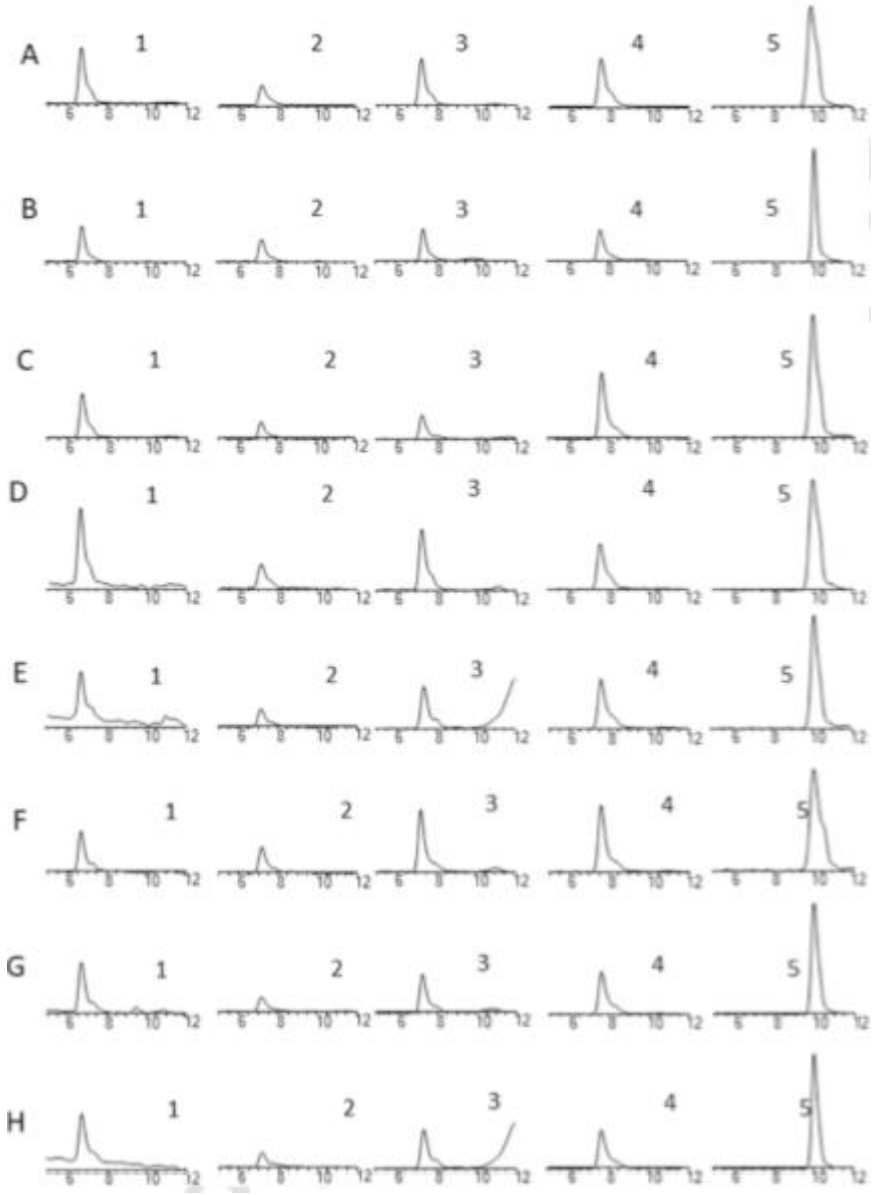

Figure 2. Extracted ion chromatograms obtained for ATS from (A) a standard solution (25 ng/mL each) and (B) oral fluid, (C) sweat, (D) urine, (E) serum, (F) breast milk, (G) hair, (H) and fingernails at the LLOQ (B-F 5 ng/mL; G-H 100 ng/g). (1) AMP (2) MA (3) MDA (4) MDMA (5) MDEA.

3.2.2. Matrix effects

Matrix effects for ATS in the seven biological matrices under study were all in the range 91-107% (Table 4), indicating that the maximum ion suppression or enhancement was 9% and 7%, respectively, and that there was absence of interfering components according to

standard guidelines [36, 37]. The mean slope values of the calibration curves obtained for each ATS from the seven biological matrices and neat standards were compared by using analysis of covariance, ANCOVA [38]. The values obtained for the level of significance of the null hypothesis (P-values) were 0.1701, 0.0810, 0.2723, 0.4191 and 0.2114 for AMP, MA, MDA, MDMA and MDEA, respectively. These values were above the P-value (0.05) for the level of confidence of 95%, so it can be concluded that significant differences did not exist among the slopes of each of the eight groups of calibration curves (i.e. seven biological matrices and neat standards), and therefore, the method was matrix-independent. Thus, solvent calibration can be used for the quantification of ATS in the intended matrices, that saving costs and increasing simplicity and sample throughput.

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Table 4. Evaluation of matrix effects (ME, %) and analytical recoveries for the determination of ATS in different biological matrices

Sample	[a]ATS Concentration	([b]Mean recovery ± SD) %					([c]ME) %				
		AMP	MA	MDA	MDMA	MDEA	AMP	MA	MDA	MDMA	MDEA
Oral fluid	5	100±6	92±6	106±4	101±3	89±4					
	25	89±3	95±3	92±3	97±3	89±3					
	250	101±3	99±3	101±3	102±3	95±3					
							92	95	107	105	102
Sweat	5	115±1	106±3	102±3	94±1	106±1					
	25	111±3	92±2	89±3	89±3	93±1					
	250	89±1	103±2	87±1	88±1	106±1					
							92	93	92	101	95
Urine	5	103±5	87±5	88±4	110±3	90±4					
	25	88±2	89±2	90±3	89±2	97±1					
	250	100±2	101±2	102±1	100±2	96±1					
	[c]50	97±3	103±2	104±4	96±4	97±1					
	[d]150	101±5	98±3	97±4	100±3	105±1					
							98	95	91	91	92
Serum	5	104±6	99±3	109±1	98±3	104±2					
	25	110±4	110±3	102±5	98±1	104±2					
	250	89±2	89±2	88±1	89±1	92±1					
							97	93	103	104	101
Breast milk	5	88±2	93±5	92±4	100±6	113±1					
	25	93±1	108±4	99±4	107±1	103±4					
	250	99±1	92±6	92±4	89±3	100±1					
							98	98	100	95	97
Hair	100	85±3	99±7	95±2	105±2	88±2					
	1000	90±3	89±3	93±5	95±2	90±1					
	10000	90±1	109±2	97±2	87±1	104±2					
							95	98	102	97	94
Fingernail	100	113±2	88±2	103±1	107±1	99±1					
	1000	94±2	101±5	89±3	91±3	94±1					
	10000	89±2	93±1	92±4	90±2	107±3					
							93	98	100	96	93

[a] Concentration in samples: ng/mL for oral fluid, sweat, urine, serum and breast milk and ng/g for hair and fingernail
[b] n=10
[c] Dilution factor (1:100)
[d] Dilution factor (1:50)
[e] Calculated by dividing the mean of slopes obtained from ten different lots of each type of sample by the mean of slopes obtained from five neat standard calibrations.

3.2.3. Precision

The within- and between-run coefficients of variation for ATS in pools of fortified biological samples are summarized on Table 5. Coefficients of variation for ATS were in the interval 0.3-9.7% at the LLOQ values and they ranged from 0.1 to 9.3% at medium and high concentrations. These values were in good agreement with international guidelines that set CVs lower than 20% at LLOQ and below 15% for the rest of concentrations [36, 37].

3.2.4. Accuracy

The accuracy of the method was checked by the evaluation of ATS recoveries in blank samples spiked at the LLOQ, medium and high concentration. Because the concentration of ATS in urine can be very high after oral administration (e.g. around 6000 ng/mL for MA [39], it was checked if sample dilution affected the accuracy of the method. Table 6 4 shows the results obtained for the different matrices. Recoveries were in the interval 85-115% for LLOQ (5 ng/mL and 100 ng/g) and 87-111% for higher concentrations. All recoveries, including diluted urines, resulted in acceptable ranges according to standard guidelines [36, 37].

The accuracy was also assessed by analyzing available RMs for serum, urine and hair. Nominal values for ATS in the RMs were at low level in serum (25 ng/mL), at -25% of the recommended cut-off by EWDTS for a confirmation test in urine (150 ng/mL) and at relevant concentrations for ATS in hair (in the range 428-1740 ng/g). All RMs contained many other illicit drugs at relevant concentrations (e.g. 23, 50 and 10 drugs in serum, urine and hair, respectively). Table 7 6 shows the results obtained, expressed as the mean concentration and recoveries found for ATS. Recoveries were in the interval 92-113%, that proving the capability of the method to give accurate results in the presence of other drugs.

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Table 5. Within- and between-run coefficients of variation (CV%) for the determination of ATS in different biological matrices

Sample	[a]ATS concentration	[b]Within-run					[d]Between-run				
		AMP	**MA**	**MDA**	**MDMA**	**MDEA**	**AMP**	**MA**	**MDA**	**MDMA**	**MDEA**
Oral fluid	5	1.4	5.0	3.3	4.5	1.9	4.5	5.3	8.5	7.2	9.7
	25	2.9	3.3	3.8	4.0	3.2	6.6	5.5	9.3	6.1	7.2
	250	2.4	1.2	1.7	1.3	2.6	3.5	1.7	7.2	2.1	6.6
Sweat	5	2.0	3.2	2.6	2.2	1.3	1.8	1.3	2.1	1.5	0.3
	25	2.0	1.3	2.3	1.3	0.4	1.8	1.3	2.1	1.5	0.3
	250	1.3	1.5	0.8	0.4	2.4	1.1	1.3	0.7	0.4	2.8
Urine	5	3.9	4.3	1.8	0.7	5.4	5.1	4.3	2.1	0.8	5.2
	25	2.2	1.5	1.7	0.8	1.1	1.1	2.8	1.5	2.1	0.9
	250	2.1	0.6	0.8	0.6	0.6	0.6	1.9	0.8	0.8	0.6
Serum	5	6.7	6.2	2.6	1.3	5.8	7.3	5.2	2.2	1.1	4.9
	25	4.1	2.3	4.0	1.2	1.8	3.4	2.0	3.4	1.0	1.5
	250	2.7	1.8	1.3	0.6	1.4	2.3	1.5	1.1	0.5	1.2
Breast milk	5	1.5	4.2	2.6	3.9	1.1	0.6	1.9	2.4	1.1	3.0
	25	0.5	1.8	2.7	1.0	3.0	0.6	1.9	2.4	1.1	3.0
	250	0.4	2.9	3.3	1.3	0.1	0.4	3.1	3.0	1.4	0.3
Hair	100	2.4	4.3	1.6	1.0	1.3	4.0	6.1	1.6	1.6	1.7
	1000	2.4	3.0	4.4	0.9	2.2	2.8	8.8	4.8	1.1	2.9
	10000	0.4	0.5	0.6	0.7	0.2	0.8	4.0	1.7	1.1	0.3
Fingernail	100	5.0	3.3	2.0	3.3	1.0	5.7	3.4	2.0	3.2	1.6
	1000	1.3	3.9	1.9	2.7	0.9	1.3	3.7	1.8	2.5	1.1
	10000	1.2	1.1	3.9	1.7	0.7	1.4	1.1	3.4	1.6	1.1

[a] It refers to concentration in samples. Units are ng/mL for oral fluid, sweat, urine, serum and breast milk and ng/g for hair and fingernail

[b] 7 replicates for each concentration

[c] 7 replicates for each concentration on four different days

Table 6. Concentrations and recoveries (R) found for ATS, along with the respective standard deviations (SD), in the analysis of reference materials

ATS	[a]Urine		[b]Serum		[c]Hair	
	[d]Concentration ± SD (ng/mL)	R± SD (%)	[d]Concentration ± SD (ng/mL)	R±SD (%)	[d]Concentration ± SD (ng/g)	R±SD (%)
AMP	142±3	95±2	24.5±0.5	98±2	1099±32	94±3
MA	163±6	109±4	26.2±0.3	105±1	868±8	109±1
MDA	150±4	100±3	24.3±1.5	97±6	484±5	113±1
MDMA	159±4	106±3	24.3±0.5	97±2	1844±17	106±1
MDEA	144±3	96±2	28.0±0.5	112±2	542±6	92±1

[a] Medidrug® WDT Confirm U -25%, nominal concentration for each ATS 150 ng/mL
[b] Medidrug® DOA-I S low, nominal concentration for each ATS 25 ng/mL.
[c] DHF 2/12-A HA, nominal concentrations: AMP (1170 ng/g), MA (797 ng/g), MDA (428 ng/g), MDMA (1740 ng/g) and MDEA (589 ng/g).
[d] n=5

3.3. Analysis of ATS positive urine samples

The method was applied to five urine samples, which tested positive for ATS by immunoassay (Dimension, Siemens). This assay has cutoffs of 300, 280 and 290 ng/mL for MDMA, MDA and MDEA, respectively. The presence of MDA was confirmed for all samples at concentrations of 5.45400 ± 0.5500, 3.83800 ± 0.1100, 2.32300 ± 0.2200, 2.62600 ± 0.2200 and 4.24200 ± 0.5500 mg/Lng/mL. No other ATS were detected above the LLOQ. The EICs obtained for the five samples analyzed are shown in Figure 3.

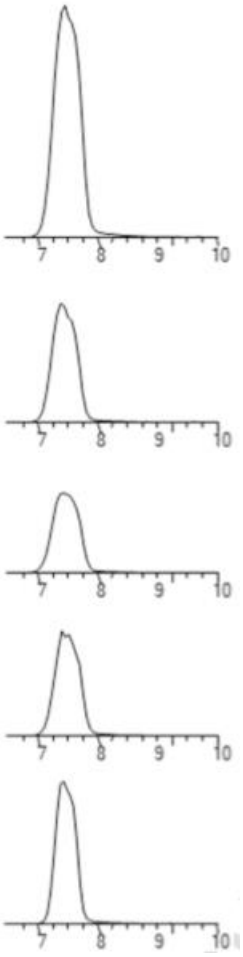

Figure 3. Extracted ion chromatograms (EIC) obtained for MDA quantification transition from a set of five anonymized ATS-positive urine samples

4. CONCLUSION

SUPRAS-RAM have proved here to be a suitable strategy to effectively extract ATS and remove matrix effects in LC-MS/MS for the quantification of these drugs in up to seven different biological matrices. The use of SUPRAS-RAM resulted in a truly universal, fast, cheap and reliable sample treatment platform for the control of ATS abuse. To the best of

our knowledge, this is the first matrix-independent platform able to deal with several biological fluids and solids in matrices of forensic and clinical interest. It is anticipated that this methodology could be easily extended to the bioanalysis of other drugs and medicaments.

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Declarations of interest: none

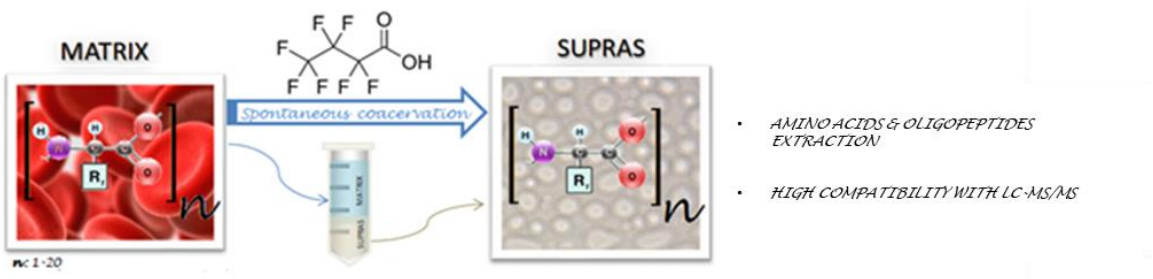
Chapter 6

Exploring polar hydrophobicity in organized media for extracting oligopeptides: application to the extraction of opiorphin in human saliva

Exploring polar hydrophobicity in organized media for extracting oligopeptides: application to the extraction of opiorphin in human saliva

Francesca Accioni, D. García-Gómez, Soledad Rubio

To be Submitted to Analytica Chimica Acta



ABSTRACT

Supramolecular solvents are gaining momentum as extractants of compounds of interest from complex matrixes such as foodstuff and biological and environmental samples. However, their powerful extraction mechanism, based on multiligand ability for solute binding, fails when applied to very polar compounds, hindering their applicability to the extraction of metabolites. In this work, we introduce the synthesis, characterization and application of a new kind of SUPRASs formed by heptafluorobutyric acid (HFBA). The polar hydrophobicity of this perfluorinated acid results in a SUPRAS, which coacervates at acidic pHs, that shows a great capability to extract amino acids and oligopeptides (recoveries in the range 81-105%) with nonpolar alkyl, cyclic or aromatic side chain substituents (with log D > -3.62). To further demonstrate the potential of this novel SUPRAS, an analytical methodology for the determination of opiorphin in real saliva samples was developed and fully validated. The HFBA-based SUPRAS was synthetized in situ from 950 μL of stabilized saliva, by the addition of 150 μL of HFBA and 400 μL of HCl 37% (v/v). The resulting SUPRAS was directly injected into a LC-MS/MS system for further quantification. Quantitative recoveries in the range of 87-110% were obtained with relative standard deviations below 20%. The HFBA-based SUPRAS is, therefore, capable of efficiently extracting opiorphin from saliva samples and shows a high potential for the determination of several amino acids and oligopeptides from biological samples.

KEYWORDS Supramolecular solvents; amino acids; oligopeptides; opiorphin; heptafluorobutyric acid.

1. INTRODUCTION

Polar hydrophobicity is a term related to fluorinated compounds that refers to the apparent discrepancy between the high polarity of the C-F bond and the pronounced compound hydrophobicity [1]. Polar hydrophobicity relies on two properties of C-F bonds; on the one hand, they are highly dipolar, so they can interact with ionic or dipolar groups by electrostatic (dipole-dipole or charge-dipole) interactions. On the other hand, C-F bonds are relatively non-polarizable, which reduces overall molecular polarizability, thus increasing compound lipophilicity [2].

Lipophilicity of organofluorine compounds has been extensively exploited in medical applications, where incorporation of fluorine into biologically active compounds can alter drug metabolism or enzyme substrate recognition and may improve drug transport across the blood brain barrier and oral bioavailability [3]. Likewise, it is of interest for the production of stain- and water-repellent surfaces [4].

Regarding the C-F bond, it is still a matter of debate whether it can stablish strong polar interactions [1, 5]. Thus, it has been proved that C-F bonds act as hydrogen bond acceptors in the gas phase but these interactions are of minimal importance in polar solvents such as alcohols, amines or water [6]. The reasons given for the different behavior of C-F bonds in the liquid and gas phases is that their low polarizability makes time-dependent interactions (e.g. dipole-induced dipole, ion-induced dipole and dispersion) not as favorable as they are for polar solvents [7]. On the other hand, a growing body of evidence indicates that the energies of electrostatic interactions of the C-F bond dipole can be substantial in the solid state, where C-F…H-C interactions have been proposed for crystal engineering design [8]. Likewise, the existence of C-F…M bonds, where M is an alkali metal cation, has been

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demonstrated in appropriately organized systems such as macrocyclic fluorinated ligands, even in polar solvents [9, 10].

This work was intended to explore the potential of polar hydrophobicity of fluorinated compounds in the analytical extraction of high polar organic cations. For this purpose, the tailoring of a proper organized system in polar solvents was undertaken with the aim of maximizing C-F…M interactions.

In order to design a proper organized system, a fluorinated amphiphile (i.e. heptafluorobutyric acid, HFBA) was selected as a model compound and self-assembly as a structure-directing process. Self-assembly, the phenomenon by which isolated components organize autonomously and spontaneously into ordered and/or functional structures [11], has become one of the most widespread and powerful strategies for the production of advanced functional supramolecular materials [12]. However, this phenomenon remains almost unexplored for the production of tailored solvents, although self-assembly has already proved an invaluable strategy for the synthesis of tailored supramolecular solvents (SUPRAS) that feature restricted access properties (SUPRAS-RAM) [13]. These tailored solvents have found multiple applications for efficient extraction of contaminants in environmental, biological and food samples while removing matrix effects in LC-MS/MS by chemical and physical mechanisms [14, 15, 16, 17, 18]. So, it was considered that fluorinated-based supramolecular solvents could be proper organized systems for maximizing C-F electrostatic interactions.

In this paper, the synthesis and characterization of heptafluorobutyric acid-based supramolecular solvents were undertaken and their suitability for extracting polar organic cations was evaluated by studying the extraction efficiency for different oligopeptides

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containing from 2 to 5 amino acids. Currently, more than 15000 oligopeptides from more than 2200 biological species are known and a large number of them (e.g. enkephalins, neurotensins, angiotensins, somatostantins, etc.) have a wide spectrum of functional activities as regulators of the nervous, endocrine and immune systems [19]. Depending on the amino acid residues making up the oligopeptides, they may establish ionic, ion-dipole, dipole-dipole and/or stacking interactions. A remarkable property of oligopeptides, compared to proteins, is the predominance of positively charged amino acid residues, so they are valuable models as polar organic cations [20].

In order to check the ability of the proposed approach to work in real world, it was applied to the extraction of opiorphin in human saliva prior to its quantification by LC-MS/MS. Opiorphin, a pentapeptide first isolated in 2006 from human saliva, is a natural enkephalin with a painkiller effect greater than morphine but with less dependence and addiction [21]. It has been recently found to be part not only of human saliva (76-237 ng mL-1 in basal conditions, 24-1091 ng mL-1 under stimulation) but also of bloodstream (0.1-3.4 ng mL-1), urine (1-27 ng mL-1), semen (3-31 ng mL-1), breast milk (3-23 ng mL-1) and tears (<2-1109 ng mL-1) [22, 23]. These values were determined by a tedious and laborious method involving treatment with a strong chelating agent, solid-phase extraction, freeze-drying and/or reversed-phase chromatography cleanup and a competitive ELISA assay [24, 25]. To the best of our knowledge, only a LC-MS/MS method has been developed for the quantification of opiorphin in human samples, specifically saliva- a biofluid in which qualitative changes can provide diagnostic information [26]. The method is based on protein precipitation, freeze-drying and reversed phase chromatography-electrospray MS/MS quantification. However, the need for using the standard addition method for reliable quantification lengthens total analysis time. Therefore, a quick and easy method based on SUPRAS extraction would be a useful tool for further studies regarding opiorphin, especially because recent studies have shown that the levels of circulating opiorphin may be

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upregulated or downregulated by different human pathological states. Below the most relevant results are discussed.

2. EXPERIMENTAL

2.1. Chemicals and reagents

The twenty proteinogenic amino acids (Glycine (Gly), Alanine (Ala), Valine (Val), Leucine (Leu), Isoleucine (Ile), Methionine (Met), Tryptophan (Trp), Phenylalanine (Phe), Proline (Pro), Serine (Ser), Threonine (Thr), Cysteine (Cys), Tyrosine (Tyr), Asparagine (Asn), Glutamine (Gln), Aspartic acid (Asp), Glutamic acid (Glu), Lysine (Lys), Arginine (Arg) and Histidine (His)), six dipeptides (Ala-Phe, Phe-Val, Ala-Ala, Glu-Glu, Phe-Gly hydrate and Ala-Tyr), and a tripeptide (Arg-Gly-Asp) were all purchased in Sigma-Aldrich (Barcelona, Spain). Standards of Opiorphin trifluoroacetate salt (OPI) and Arg-Phe acetate salt were obtained from Bachem (Bubendorf, Switzerland). Protease Inhibitor Cocktail, acetonitrile, formic acid, hydrochloric acid 37% (HCl) and Heptafluorobutyric acid 98% (HFBA) were also purchased from Sigma Aldrich. Type I water was obtained from a purification system (Millipore, Madrid Spain).

Stock solutions for each compound (100 μg mL-1) were prepared in type I water and methanol (30:70 v/v) and kept at -20 °C. Calibration standards and working solutions were made daily by diluting the proper amount of stock solutions in water.

2.2. Instrumentation

LC-MS/MS analyses were run in an Agilent Technologies 1200 series LC coupled to a 6420 triple quadrupole mass spectrometer with an electrospray ionization source (ESI) (Waldbronn, Germany). Chromatographic separation of amino acids, dipeptides and tripeptides was carried out on a Luna CN 100 Å column (100 x 2 mm i.d., particle size 2 μm) from Phenomenex. Separation of opiorphin was performed on an InfinityLab Poroshell 120 HILIC column (2.1 x 150 mm i.d., particle size 4 μm) from Agilent. For the synthesis of SUPRAS, a Reax Heidolph vortex mixer (Schwabach, Germany) and an MPW -350R centrifuge from MPW Med- Instruments were used (Warchaw, Poland). A Karl Fischer coulometric titrator from Metrohm (Herisaus, Switzerland) was used to determine SUPRAS composition. A digital caliper was used for measuring the volume of SUPRAS obtained under different experimental conditions. A light microscope Leica DM 500 B (Heerbrugg, Switzerland) was used to reveal the structure and the dimension of the micelles forming the SUPRAS.

2.3. Procedures

2.3.1. Extraction efficiency studies

950 μL of water, fortified at 0.1 μg mL-1 (for each oligopeptide or amino acid), 400 μL of HCl 37% (v/v) and 150 μL of HFBA were added to 1.5-mL Eppendorfs, vortex-shaken for 10 minutes and centrifuged at 21125g for 5 minutes. SUPRAS were obtained in the lower layer since they are denser than the aqueous equilibrium solution. HFBA-based SUPRAS was then extracted by means of a pipette, transferred to a vial and directly injected into the LC-MS/MS system for further analysis. Chromatographic separation of amino acids and oligopeptides was achieved in the cyano column by using an isocratic mobile phase formed by a 0.1% formic acid aqueous solution (solvent A) and Acetonitrile (solvent B) 70:30 (v/v) respectively. The injection volume was set at 10 μL.

2.3.2. Phase diagram and SUPRAS composition and volume

A ternary phase diagram was constructed by mixing HCl 37% (v/v), HFBA and water at different mass percentages (w/w). The SUPRAS formation region was delimited by visual inspection of liquid-liquid phase separation in the mixed solution after centrifugation. Both SUPRAS composition and volume was determined into the SUPRAS region at 40 different mass percentages of the ternary mixture. Water and, by extension, HCl contents were measured by the use of a Karl Fischer Coulometric titrator. The amount of HFBA was then

calculated by difference. The volume of SUPRAS was obtained by measuring its height in the 1.5-mL Eppendorfs with a digital caliper.

2.4. Determination of Opiorphin in saliva

2.4.1. Samples collection

Oral fluid was collected from volunteers in agreement with The Declaration of Helsinki. For saliva sampling, some specifics were followed: volunteers were asked not to drink, eat, smoke or chew gum one hour before collection. After the sampling, saliva was immediately stabilized, analyzed and/or stored at -20°C for further analysis. In order to prevent the rapid enzymatic degradation of OPI in oral fluid, a Protease Inhibitor Cocktail containing 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) at 2 mM, Aprotinin at 0.3 µM, Bestatin at 116 µM, E-64 at 14 µM, Leupeptin at 1 µM and EDTA at 1 mM was immediately added to the sample. Blank sample matrices, useful for method development, were obtained by the same process but skipping the inhibitor cocktail.

2.4.2. HFBA-based SUPRAS extraction

Taking advantage of the high amount of water in saliva, HFBA-based SUPRASs were synthetized in situ in the sample. For this purpose, 950 µL of stabilized saliva were added to 150 µL of HFBA and 400 µL of HCl 37% (v/v) in 1.5-mL Eppendorfs. The mixture was vortex-shaken for 10 minutes and centrifuged at 21125g for 5 minutes and the SUPRAS (around 180 µL) was obtained in the lower layer (Figure 1). After that, a SUPRAS aliquot was injected into the LC-MS/MS system for quantification of opiorphin.

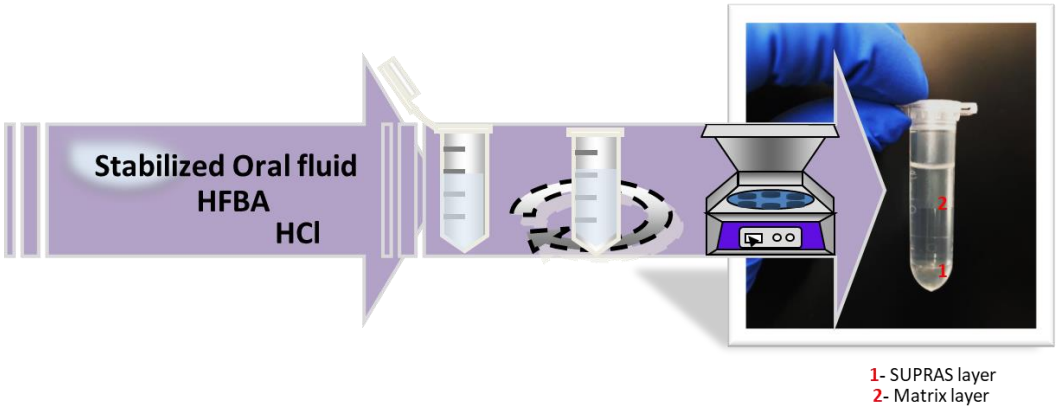

Figure 1. Schematic representation of HFBA-based SUPRAS synthesis in saliva.

2.4.3. LC separation and MS/MS quantification

For the determination of Opiorphin in saliva, the chromatographic separation was carried out in the HILIC column by an isocratic mobile phase formed by a 0.1% formic acid aqueous solution (solvent A) and Acetonitrile (solvent B) at a proportion of 30:70 (v/v), respectively. MS/MS parameters for all the analytes studied in this work were optimized by direct infusion and the most abundant collision-induced fragments were considered for quantification (Table S1). ESI source parameters were as follows: source gas: 50 psi of N2 at 350 °C; capillary voltage: +4000V. Calibration curves for opiorphin were prepared by spiking water or stabilized saliva with Opiorphin (0, 10, 20, 50, 100, 200, 500, 1000 ng mL-1) and injecting the afterwards synthetized SUPRAS into the LC-MS/MS system.

3. RESULTS AND DISCUSSION

3.1. HFBA-based SUPRAS synthesis and characterization

HFBA is a strong acid (pKa 0.04) [27] with high solubility in water (~214 g L-1) and one of the smallest micelle-forming molecules known (critical aggregation concentration, cac, around 1M) [28]. In order to produce HFBA-based SUPRAS in aqueous solution, the HFBA micellar aggregates should grow through self-assembly processes until giving liquid-liquid phase separation (i.e. coacervation). Aggregation of amphiphiles in solution is a start-stop process; usually solvophobicity drives aggregation while the stop process emanates from head group-head group repulsion [29]. So, decrease of the repulsion of the head groups of HFBA, highly ionized in aqueous solution, was tried by addition of hydrochloric acid.

Table 1 shows the initial compositions for the ternary mixture HFBA, water and concentrated hydrochloric acid, expressed as mass percentages, at which SUPRAS formed. It should be highlighted that at least a molar ratio of 1:1 regarding HCl:HFBA was necessary to produce a complete coacervation of the amphiphile. On the other hand, the maximum concentration easily achievable for concentrated HCl (30%, w/w) was set as the upper limit for this component.

According to the results obtained (Table 1), the chemical composition of the SUPRAS, expressed as mass percentage, kept quite constant and ranged in a much lower interval (2-5%

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HCl, 9-27% H2O and 70-86% HFBA) compared to the composition of the synthetic solutions (5-30% HCl, 30-80% H2O and 10-55% HFBA). Incorporation of the amphiphile to the SUPRAS was quantitative (average value 107±6%) without regard to the initial ternary composition. This unexpected property for an amphiphile featuring a cmc of 1M is unusual and beneficial in relation to its extraction capability since no amphiphile is lost in the equilibrium solution. So, partition of analytes to the SUPRAS should be favored.

Table 1 shows the SUPRAS main properties, including composition, for those initial conditions from which an unequivocal amount of SUPRAS was formed after centrifugation.

Table 1. Characterization of SUPRAS formed from different initial ternary mixtures

INITIAL CONDITIONS			SUPRAS COMPOSITION AND MAIN PROPERTIES						
HCl / %	**HFBA / %**	**Water / %**	**Water / %**	**HCl / %**	**HFBA / %**	**HFBAin SUPRAS Incorporation / %**	**Density / g/cm³**	**SUPRAS volume / µL**	**Solution/ SUPRAS ratio**
5	20	75	26	2	72	109	1.51	331	2.0
10	**10**	**80**	**27**	**3**	**70**	**116**	**1.50**	**181**	**3.5**
10	15	75	23	3	74	110	1.52	241	2.4
10	20	70	22	3	75	107	1.53	309	1.7
10	25	65	21	3	76	106	1.53	373	1.3
10	30	60	19	3	78	109	1.54	447	1.0
10	35	55	21	4	75	103	1.53	513	0.7
10	40	50	14	3	83	111	1.56	562	0.6
15	10	75	22	4	74	116	1.52	170	2.9
15	15	70	18	4	78	114	1.54	231	1.9
15	20	65	17	4	79	115	1.55	307	1.3
15	25	60	15	4	81	101	1.56	328	1.1
15	30	55	15	4	81	95	1.56	369	0.8
15	35	50	15	5	80	104	1.55	444	0.7
15	40	45	13	4	83	99	1.56	500	0.4
15	45	40	12	5	83	105	1.57	592	0.2
15	50	35	10	4	86	104	1.58	630	0.2
15	55	30	10	5	85	101	1.57	685	0.1
20	10	70	14	4	82	107	1.56	163	2.7
20	15	65	14	4	82	111	1.56	214	1.8
20	20	60	12	4	84	102	1.57	255	1.4
20	25	55	10	4	86	100	1.58	303	1.0
20	30	50	11	4	85	97	1.57	360	0.7
20	35	45	18	6	76	117	1.53	413	0.5
20	40	40	9	4	87	102	1.58	492	0.3
25	10	65	11	4	85	116	1.57	144	2.7
25	15	60	11	4	85	107	1.57	198	1.7
25	20	55	10	4	86	115	1.58	278	1.1
25	25	50	10	5	85	114	1.57	346	0.7
30	10	60	10	5	85	112	1.57	137	2.5
30	15	55	10	5	85	105	1.57	195	1.5

In bold, SUPRAS selected as extractant
Percentages are expressed as w/w

SUPRASs of very similar composition were obtained by increasing the concentration of HFBA in the synthetic solution while keeping constant that of HCl (Table 1). The volume for these SUPRAS progressively increased, as usual, when the concentration of HFBA did. On the other hand, less aqueous SUPRASs were obtained for ternary mixtures in which HFBA kept constant and HCl progressively increased. Solution/SUPRAS phase ratios ranged in the interval 0.1, corresponding to the highest concentration of HFBA tested (i.e. 55%), and 3.5, corresponding to that mixture containing the lowest concentration of HFBA (10%) and HCl (10%). So the latter conditions are more favorable for extraction purposes and they were selected for further studies.

Figure 2 shows the micrographs obtained by light microscopy for the SUPRAS synthesized from 10% HCl, 10% HFBA and 80% water. These micrographs clearly show that it consists of coacervate droplets that keep as individual entities. So the high superficial area of SUPRAS facilitates solute mass transfer in extraction processes.

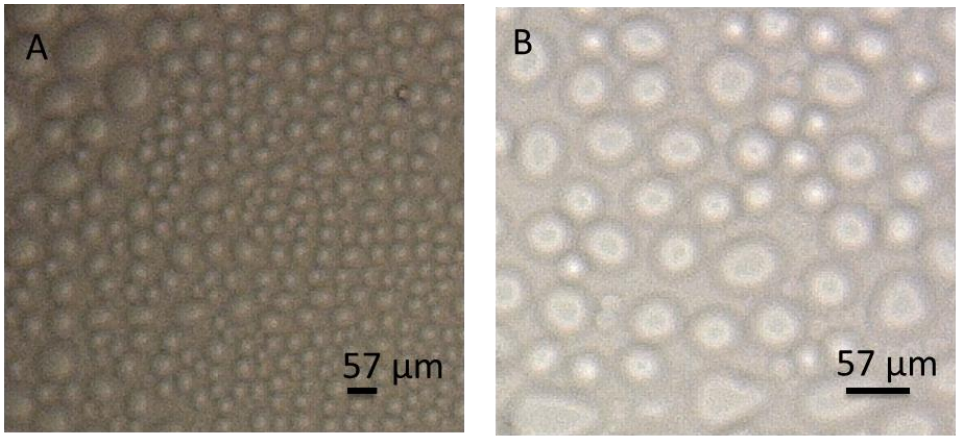

Figure 2. Light microscopy microphotographs of the HFBA-based SUPRAS formed by an initial mixture of 10% HCl, 10% HFBA and 80% water.

3.2. HFBA-based SUPRAS extraction of oligopeptides

In order to check the suitability of the selected HFBA-based SUPRAS for extracting oligopeptides, the extraction of seven dipeptides, one tripeptide and one pentapeptide (opiorphin), featuring different amino acid residues, was investigated in aqueous solutions according to the procedure specified in Section 2.3.1. As it is shown in Table 1, recoveries were all in the range 81-105 %, except for the dipeptide Glu-Glu and the tripeptide Arg-Gly-Asp.

Table 2. Extraction of oligopeptide with HFBA-based SUPRAS

OLIGOPEPTIDES	RECOVERY / %
PHE-VAL	105
ALA-PHE	98
ALA-ALA	86
PHE-GLY	84
ALA-TYR	81
ARG-PHE	84
GLU-GLU	8
ARG-GLY-ASP	19
GLN-ARG-PHE-SER-ARG	94

Because of the large number of natural oligopeptides of interest in biology and food science, the extraction of the twenty standard amino acids with HFBA-based SUPRAS was investigated in order to shed light on the extraction behavior of the selected oligopeptides. Experiments were carried out according the procedure described in section 2.3.1. Table 3 shows the recoveries obtained as well as some structural and physico-chemical characteristics of amino acids in order to help understanding interactions with the SUPRAS.

Table 3. Recoveries for amino acids extracted with HFBA-based SUPRAS and some structural and physico-chemical properties

Amino acid abbreviation	Recovery	Side chain substituent	Log D	Residue level hydrophobicity	Isoelectric point
		Nonpolar aliphatic or cyclic			
Ala	81	Alkyl	-3.62	1.0	6
Val	88	Alkyl	-2.75	-2.0	6
Leu	87	Alkyl	-2.27	-3.5	6
Ile	105	Alkyl	-2.26	-3.5	6
Pro	105	Rigid cyclic structure	-3.07	-4.0	6.3
Met	86	Sulphide	-2.79	-1.0	5.7
		Aromatic			
Phe	98	Phenyl	-2.79	-4.0	5.5
Tyr	30	Phenolic	-3.60	-1.5	5.7
Trp	35	Indole	-2.31	-3.0	5.9
		Polar uncharged			
Ser	30	Hydroxyl	-4.44	3.5	5.7
Thr	38	Hydroxyl	-4.07	2.0	5.6
Gln	30	Amide	-4.60	5.0	5.7
Cys	10	Thiol	-2.85	3.5	5.0
Asn	22	Amide	-4.90	6.5	5.4
		Negatively charged			
Glu	30	Carboxylic acid	-3.98	6.0	3.2
Asp	22	Carboxylic acid	-4.11	7.5	2.8
		Positively charged			
Lys	17	Amino	-4.78	5.0	9.7
Arg	17	Guanidine	-5.70	14.5	10.8
His	22	Imidazole ring	-5.34	5.0	7.6
Gly	30	***None***	-3.97	2.5	6

As can be seen, the behavior of amino acids towards HFBA-based SUPRAS extraction can be grouped in two categories: those extracted with quantitative yields (>80%) and those featuring medium-poor yields (10-38%). Because all amino acids were positively charged at the experimental conditions under which extraction was undertaken (see isoelectric points (Table 3), the different behavior found can be rationalized according to the type substituent on the amino acid side chain. Thus, those amino acids with alkyl or phenyl substituents were all quantitatively extracted. However, neutral amino acids with side chains containing hydroxyl, sulfur or nonbasic nitrogen, or those positively (basic) or negatively (acidic) charged were extracted with medium-poor yields. The only exception to this grouping was Met; the side chain contains sulfur but it is quantitatively extracted.

In an attempt to correlate the behavior of amino acids towards HFBA-based SUPRAS extraction with the hydrophobicity of amino acid residues, two hydrophobicity scales (log D and residue level hydrophobicity) were considered (Table 3). The first one is based on the partition of amino acids between an oil phase and a water phase (Log D) while the second one allows for the depiction of the polar and non-polar moieties within each amino acid

residue and it is calculated by a weighted sum of the atomic hydrophobicity values [30]. It is clear that those amino acid residues with positive values in the residue level hydrophobicity scale (i.e. amino acids with none, polar uncharged, positively and negatively charged side chain substituents) were extracted with medium-poor yield, while those with negatively value were quantitatively extracted. The only exceptions to this behavior were Ala, Tyr and Trp. Similar conclusions can be extracted from the log D scale; in general the more negative values correspond to the lower extraction yields. On the whole, it seems that amino acid residues having in the side chain heteroatoms able to establish hydrogen bonds (O, N) with water, will partition less favorably to the SUPRAS. This is the most probable reason why Tyr and Trp were not quantitatively extracted despite their Log D and residue level hydrophobicity values (Table 3).

Regarding extraction of oligopeptides, it was experimentally found that quantitative extraction was related to the presence into their structures of amino acid residues with nonpolar aliphatic, cyclic or phenyl substituents in the chain side (compare Tables 2 and 3). Thus, the oligopeptides containing well-extracted amino acid residues (e.g. Phe-Val, Ala-Phe, Ala-Ala) were quantitatively extracted, while those containing poor extracted amino acid residues (e.g. Glu-Glu, Arg-Gly-Asp) were poorly extracted. On the other hand, the presence of the following amino acid residues (in parenthesis) seems to be essential for good recovery of the investigated oligopeptides; Phe-Gly (Phe) Ala-Tyr (Ala), Arg-Phe (Phe), Gly-Arg-Phe-Ser-Arg (Phe). Keeping in mind the substantial hydrophobic regions present in natural oligopeptides such as neuropeptides, antimicrobial, hormones and toxins [20], it is expected that HFBA-based SUPRAS extraction can be widely applied in this field.

3.3. Determination of opiorphin in human saliva by means of HFBA-based SUPRAS and LC-MS/MS

In order to show the applicability of the HFBA-based SUPRAS proposed in this work, a novel analytical method for the determination of opiorphin in human saliva by SUPRAS extraction coupled to LC-MS/MS was developed. Table 4 shows the main analytical characteristics of such a method.

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Table 4. Analytical performance of the SUPRAS-LC-MS/MS method for the determination of opiorphin in human saliva.

		OPIORPHIN
Retention time[a] / min		1.8
Linear range / ng mL^{-1}		10-2000
Slope[b]/UA mL ng^{-1}	***Standard***	0.49±0.02
	Saliva after	0.44±0.01
	Saliva before	0.45±0.01
Extraction efficiency[c] / %		102±3
Matrix effect[d] / %		90±4
LOD[e] / ng mL^{-1}		2.7
LOQ / ng mL^{-1}		9.0
Repetibility[f] / RSD	***10 ng mL^{-1}***	14±5
	200 ng mL^{-1}	13±3
	2000 ng mL^{-1}	11±2
Reproducibility / RSD	***10 ng mL^{-1}***	19±7
	200 ng mL^{-1}	18±5
	2000 ng mL^{-1}	12±4
Accuracy[g] / %	***10 ng mL^{-1}***	110±20
	200 ng mL^{-1}	87±9
	2000 ng mL^{-1}	101±2

a. HILIC column. Mobile phase: 30% Water and 70% Acetonitrile
b. Calculated by linear regression from SUPRAS-extracted standards (*standard*) and blank saliva samples spiked after (*saliva after*) and before (*saliva before*) SUPRAS extraction
c. Calculated as the ratio of *saliva before* and *saliva after* slopes
d. Calculated as the ratio of *saliva after* and *standard* slopes
e. LOD (limit of detection) calculated as the concentration resulting in a signal-to-noise ratio of 3 (10 for LOQ).
f. Calculated by the consecutive analysis of 8 saliva samples (8 samples x 3 consecutive days for reproducibility)
g. Calculated as recoveries from spiked saliva samples not used for calibration

It should be highlighted that the extraction efficiency was ca. 100%, which confirms that the quantitative recoveries found for standards can also be achieved with real samples. Limits of detection and quantification are in the same order of magnitude of those based on other more tedious sample treatment techniques. It should also be noted that the matrix effect from other salivary compounds is almost negligible, pointing out the excellent matrix clean-up obtained using the HFBA-based SUPRAS protocol. Regarding repeatability and reproducibility, values below 20% were found for low, medium and high concentrations. Likewise, accuracies, calculated as recoveries since no certified reference materials are available, were within the 80-120% range for the whole calibration interval.

Figure 3 shows a LC-MS/MS chromatogram obtained by the analysis of unspiked human saliva. Opiorphin was detected and quantified in all saliva samples (n: 32), with values ranging from 25 to 175 ng mL^{-1} (Table S2), which is in good agreement with concentrations previously reported [22, 23].

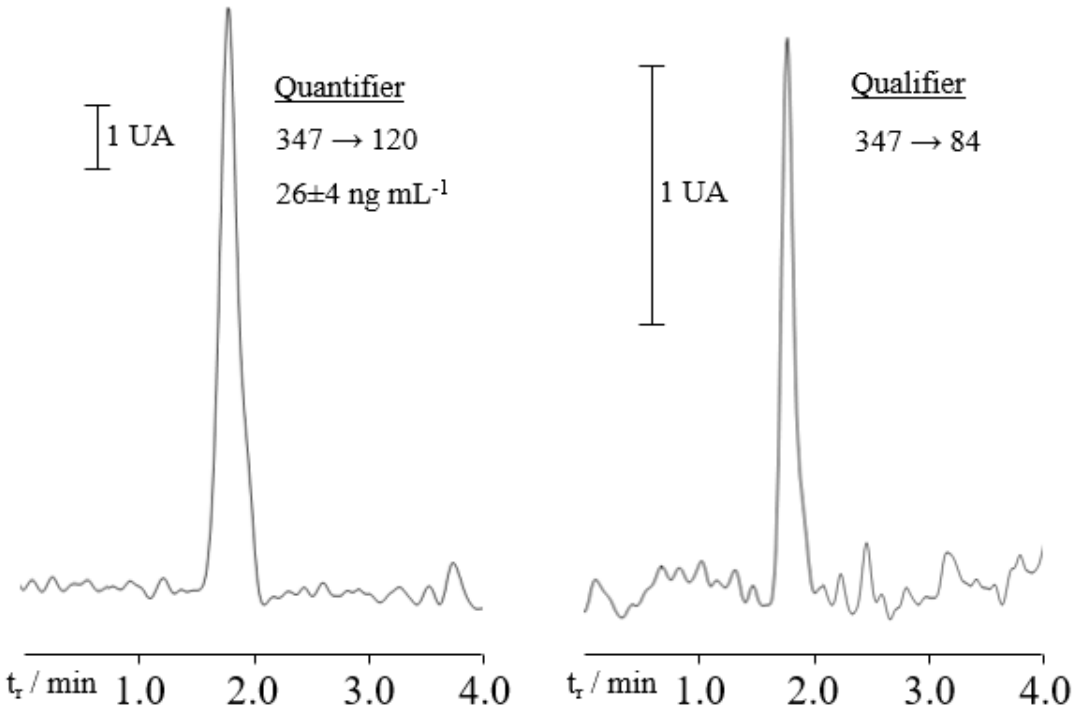

Figure 3. LC-MS/MS chromatogram obtained from a SUPRAS extract (unspiked saliva).

4. CONCLUSIONS

A new kind of SUPRASs based on perfluorocarboxylic acids has been synthetized, characterized and applied. This novel HFBA-based SUPRAS circumvents, thanks to its polar hydrophobicity, one of the main disadvantages of SUPRAS extraction: the poor recoveries accomplished when extracting vey polar charged compounds. It has been demonstrated that amino acids containing nonpolar aliphatic, cyclic and aromatic side chain substituents, in the log D interval -2.27 to -3.62, can be quantitatively extracted and that oligopeptides containing some of these amino acid residues may also be quantitatively extracted. In addition, the applicability of this new SUPRAS has been established by developing an analytical methodology for the determination of an endogenous oligopeptide in human saliva. In this sense, the HFBA-based SUPRAS has exhibited a great analytical performance in terms of accuracy, precision, sensibility and matrix clean-up and also in terms of quickness, low cost and ease of use, compared to previous reported methods. We hypothesize that this performance could be extended to several different polar cations of great importance in fields such as biology, food analysis and disease diagnosis. Furthermore, the applicability to other biological (blood, urine, plasma, etc.) and non-biological matrices (foodstuff, environmental samples, etc.) should be also explored.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

Table S1. MS/MS transitions, fragmentor voltages and collision energies for all the amino acids and oligopeptides studied.

Amino acid / oligopeptide	Precursor Ion	Product Ion	Fragmentor (V)	Collision Energy (eV)
OPI-quantitative	347 $[M+2H]^{2+}$	120	100	35
OPI-qualitative		84	100	40
OPI-qualitative		409	100	15
Arg-Phe	322	*70*	100	30
Ala-Phe	277	*84*	80	29
Phe-Val	265	*120*	104	20
Ala-Ala	161	*44*	80	17
Glu-Glu	277	*84*	80	29
Phe-Gly	223	*120*	92	12
Arg-Gly-Asp	347	*330*	100	20
Ala-Tyr	253	*182*	92	8
Gly	76	*30*	70	5
Ala	90	*44*	40	9
Val	118	*72*	75	8
Leu	86	*43*	130	20
Ile	86	*57*	130	20
Met	150	*133*	80	4
Trp	205	*188*	80	5
Phe	166	*120*	80	8
Pro	116	*70*	80	12
Ser	106	*60*	70	5
Thr	120	*74*	75	10
Cys	241	*152*	80	8
Tyr	182	*165*	80	12
Asn	133	*87*	74	5
Gln	147	*130*	70	5
Asp	134	*116*	80	4
Glu	148	*130*	80	4
Lys	147	*84*	80	16
Arg	175	*116*	110	12
His	156	*110*	80	15

Table S2. Opiorphin concentrations in human saliva (unspiked samples).

Opiorphin concentration in saliva/ ng mL^{-1}
51±6
54±6
<LOD
<LOD
78±8
81±8
81±8
49±5
100±10
81±8
99±9
170±20
<LOD
72±8
<LOD
25±4
90±9
85±9
63±6
83±8
58±6
69±7
85±9
74±8
85±9
140±10
83±8
40±5
80±8
<LOD
150±20
<LOD

Chapter 7

Restricted access volatile supramolecular solvents for single-step extraction/cleanup of benzimidazole anthelmintic drugs in milk prior to LC-MS/MS

Restricted access volatile supramolecular solvents for single-step extraction/cleanup of benzimidazole anthelmintic drugs in milk prior to LC-MS/MS

Francesca Accioni, N. Caballero-Casero, D. García-Gómez, Soledad Rubio
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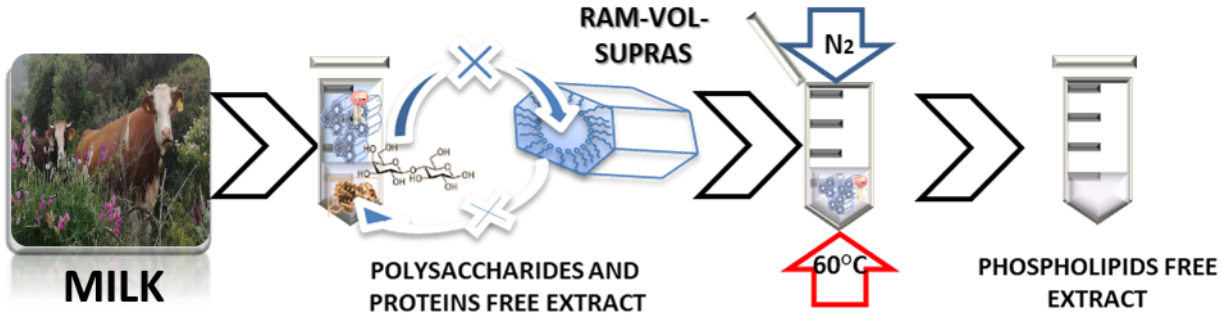

ABSTRACT

In this work, a restricted access volatile supramolecular solvent (RAM-VOL-SUPRAS) directly synthesized in milk is proposed for the first time for the simultaneous extraction and cleanup of benzimidazole anthelmintic drugs in milk meant for human consumption. The RAM-VOL-SUPRAS was formed by the self-assembly and coacervation of hexanol in tetrahydrofuran induced by the water content in milk. Benzimidazoles legislated by the European Union were quantitatively extracted (80-110%) Proteins were precipitated by the action of THF and the amphiphile, extraction of carbohydrates was avoided by a size exclusion mechanism and lipids were removed during hexanol evaporation. The analytical methodology was fully validated according to Commission Decision 2002/657/EC. Method detection limits from 0.03 to 0.14 μg L^{-1} were well below the maximum residue limits legislated in milk for these drugs, with interday precisions at maximum residue levels below 13%. This novel methodology guarantees a rapid and reliable tool for daily and routinely laboratory analyses in the field of food quality control.

KEYWORDS Supramolecular solvents; restricted access materials; anthelmintic drugs; milk; Commission Decision 2002/657/EC.

1. INTRODUCTION

Benzimidazole compounds (BDZ) belong to a heterogeneous group of drugs routinely employed in agriculture and veterinary medicine as agents for the prevention and treatment of parasite infestations [1, 2]. Because of their growing use due to their effective pharmacological activity against gastrointestinal worms, which infect animals intended for human consumption, and against flukes that contaminate vegetables, the presence of BDZ in animal-derived food products is a startling phenomenon for the possible consequences on human health (i.e., teratogenicity, congenic malformations, anemia, necrotic lymphadenopathy and pulmonary edemas) [3].Thus, monitoring BDZ in animal products has been a priority for the European Union (EU) (Commission Regulation (EU) No 37/2010) [4]. Maximum residue limits (MRLs) for foodstuffs of animal origin have been set at 100 μg kg^{-1} for albendazoles (considering ABZ-SO_2, ABZ-SO and ABZ-NH_2-SO_2 as marker residues, expressed as ABZ), and 10 μg kg^{-1} for fenbendazoles (expressed as sum of extractable residues which may be oxidized to oxfendazole sulfone) [4].

Even though many analytical methods have been developed for the detection of BDZ in foodstuff, the determination of these compounds in milk is still challenging. In fact, a detailed review of the previous works developed over the last decade for the determination of BDZ in milk by LC-MS/MS analysis (Table 1) shows that the key problem lies in the sample treatment step. In that sense, protein precipitation/ultrafiltration [5], solid phase extraction (SPE) coupled [6] or not [7] with liquid phase deposition, QuEChERS methodology [8, 9], on-line solid-phase extraction [10] and polymer monolith microextraction (PMME) [11], have been suggested as viable alternatives alternatives to classical methodologies. Recoveries (Table 1) obtained by these methods showed the highest yields when a SPE [6, 8, 10, 11] or QuEChERS [9] approach was employed, and the lowest

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[8] P. Jedziniak, M. Olejnik, J.C. Rola, T. Szprengier-Juszkiewicz, Bull. Vet. Inst. Pulawy, 59 (2015) 515-518.
[9] B. Kinsella, S.J. Lehotay, K. Mastovska, A.R. Lightfield, A. Furey, M. Danaher, Anal. Chim. Acta, 637 (2009) 196-207.
[10] D. García-Gómez, M. García-Hernández, E. Rodríguez-Gonzalo, R. Carabias-Martínez, Anal. Bioanal. Chem., 404 (2012) 2909-2914.
[11] X.Z. Hu, J.Q. Wang, Y.Q. Feng, J. Agric. Food Chem., 58 (2009) 112-119.

performing and the lowest performing when sample treatment was achieved by protein precipitation [5]. However, despite the different steps intended for interference removal, matrix-matched calibration or isotopically labelled internal standards are mandatory for reliable quantification of BDZ, that rendering methods time-consuming or expensive.

Table 1. Comparison between different samples treatment methods, published over the last decade, for the determination of anthelmintic benzimidazoles in milk by LC-MS/MS.

Sample amount	Analytes	Sample treatment	Chemicals involved in the process	Separation/ detection	Recoveries (%)	Analytical limits ($\mu g\ L^{-1}$)	References
1 g	ABZ ABZ-NH_2-SO_2 FBZ FBZ-SO_2 OFZ FBT	Protein precipitation (PPT) coupled to Ultrafiltration	2 mL of acetonitrile	LC-MS/MS (IT-ToF) (Matrix-matched calibration)	82 110 78 73 72 76	CCβ 2.8–105	(Kang et al. 2017)
0.5 mL	ABZ ABZ-SO ABZ-SO_2 FBZ FBZ-SO_2 OFZ	Solid-phase extraction (SPE) in which the composite is prepared via liquid phase deposition (LPD)	6.5 mL 20 mM phosphate buffer solution (PBS, pH 7.0) 2 mL deionized water 3.0 mL ammonium hydroxide/acetone (5:95, v/v)	LC-MS/MS (QqQ) (Internal calibration, I.S.)	76-114 92-108 92-117 73-117 97-109 90-116	LOQ 2.6-7.4	(Sun et al. 2016)
2 g	ABZ ABZ-SO ABZ-SO_2 ABZ-NH_2-SO_2 FBZ FBZ-SO_2	A modified QuEChERS method	10 mL of acetonitrile 2 g of ammonium acetate 0.150 g of magnesium sulphate 0.050 g of C18 bulk-sorbent	LC-MS/MS (QqQ) (Internal calibration, I.S.)	No reported	LOQ > 1-10	(Jedziniak et al. 2015)
2 g	ABZ ABZ-SO ABZ-SO_2 ABZ-NH_2-SO_2 OFZ FBT	Solid-phase extraction (SPE)	5 mL of 1.0 % acetic acid in acetonitrile 9 mL of 0.10 M hydrochloric acid solution. 6 mL methanol 4 mL of 10 % ammonia in acetonitrile. 1.0 mL of 0.1 % formic acid with 5 mM ammonium acetate/methanol (90:10, v/v).	UHPLC-MS/MS (QqQ) (Internal calibration, I.S.)	92-112 94-104 87-97 101-110 97-117 91-105	CCβ 12-125	(Hou et al. 2014)

2.5 mL	ABZ ABZ-SO ABZ-SO_2 ABZ-NH_2-SO_2 FBZ FBZ-SO_2 OFZ FBT	On-line solid-phase extraction	5 mL methanol, 2.5 mL of McIlvaine buffer (pH 2.5) and 0.37 g of EDTA. pH was adjusted to 7.2 with NaOH	LC-MS/MS (QqQ) (External calibration)	91-115 93-110 93-103 99-108 90-101 N.D. 99-114 87-106	LOQ 0.1-0.8	(García-Gómez et al. 2012)
0.5 mL	ABZ ABZ-SO ABZ-SO_2 ABZ-NH_2-SO_2 FBZ FBZ-SO_2 OFZ	Polymer monolith microextraction (PMME) technique	5.7 mL of 20 mM phosphate solution (pH 5.0) 0.15 mL of MeCN 0.2 mL of water	LC-MS/MS (QqQ) (Matrix-matched calibration)	75-108 81-105 83-92 81-106 79-115 81-108 82-94	LOQ 1.66-4.69	(Hu et al. 2010)
10 g	ABZ ABZ-SO ABZ-SO_2 ABZ-NH_2-SO_2 FBZ FBZ-SO_2	QuEChERS method	10 mL of acetonitrile 5 g $MgSO_4$:NaCl (4:1, w/w) 0.150 g $MgSO_4$ 0.050 g C18	LC-MS/MS (QqQ) (Internal calibration, I.S.)	104 88 98 85 70 98	LOQ 5	(Kinsella et al. 2009)

N.D.: not determined

Supramolecular solvents (SUPRAS) are nanostructured systems generated from colloidal suspensions of amphiphiles [12].Their synthesis is a spontaneous process of self-assembly and coacervation triggered by changes in environmental conditions (e.g., pH, temperature, salt or poor-solvent addition). Surfactants in SUPRAS are bound together by non-covalent interactions, which make them tailored solvents [13]. Among SUPRAS, one of the most studied alternatives are alkanols-based SUPRAS, whose synthesis is a water-induced mechanism from colloidal suspensions of an alkanol, typically hexanol, in an organic solvent (e.g. THF). In alkanol-based SUPRAS, the incorporation of amphiphiles is almost 100% and, in general, the concentration of surfactant ranges from 0.1 to 1.0 mg μL-1 [14].These SUPRAS arrange themselves in inverted hexagonal aggregates in which the -OH heads surround aqueous cavities and the hydrocarbon chains are solved in THF. Since their synthesis is spontaneously triggered by the addition of water, the amphiphile aggregation has been directly prompted in situ in aqueous biological matrices such as urine [14]. Considering this fact, and due to the high content of water in milk, this *in situ* synthetic approach is here explored for the determination of BDZ in milk.

Alkanol-based SUPRAS have outstanding properties for increasing extraction efficiency and simplifying and speeding up sample treatment [13]. Thus, their structure provides a high number of binding sites to the target analytes within regions with different polarity, which makes SUPRAS a great extractant for compounds with very different physico-chemical properties such as BDZ [12]. On the other hand, they can act as restricted access materials (RAM-SUPRAS) [14], in which low molecular weight solutes are extracted while macromolecules (e.g., interferences of complex biological matrices such as polysaccharides or proteins) are excluded by chemical and physical mechanisms [13]. In addition, it has been demonstrated that interferences such as phospholipids can be totally removed by evaporating the SUPRAS extract until dryness and re-dissolving in the proper solvent. SUPRAS that combines these two processes are known as restricted access volatile supramolecular solvents (RAM-VOL-SUPRAS) [14].

In this work, a hexanol-based RAM-VOL-SUPRAS was employed for the successful extraction of eight BDZ (i.e., Albendazole and Fenbendazole families) in milk for human

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[13] A. Ballesteros-Gómez, S. Rubio, Anal Chem., 84 (2011) 342-349.
[14] J.A. Salatti-Dorado, N. Caballero-Casero, M.D. Sicilia, M.L. Lunar, S. Rubio, Anal. Chim. Acta, 950 (2017) 71-79.

consumption. Separation and detection of the target compounds was achieved using LC-MS/MS analysis. The method was fully validated according to the Commission Decision 2002/657/EC [15].

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Standards of albendazole (ABZ), albendazole sulfone (ABZ-SO2), albendazole sulfoxide (ABZ-SO), albendazole-2-aminosulfone (ABZ-NH_2-SO_2), fenbendazole (FBZ), fenbendazole sulfone (FBZ-SO2), oxfendazole (OFZ), and febantel (FBT) were purchased from Sigma-Aldrich (Steinheim, Germany). Methanol, acetonitrile, dimethyl sulfoxide, ammonium formate, citric acid and disodium hydrogen phosphate were also purchased from Sigma Aldrich. Tetrahydrofuran (THF) was supplied by Panreac (Barcelona, Spain). 1-hexanol was purchased from VWR-Prolabo (Bois, France). Type I water was obtained from a Milli-Q purification system (Millipore MA, USA).

Stock solutions for each compound (500 μg mL^{-1}) were prepared in dimethyl sulfoxide and kept at -20 °C. Calibration standards up to 500 μg L^{-1} (n=11) and working solutions were made daily by diluting the proper amount of stock solutions in McIlvane buffer at pH 7.2 containing 10% MeOH (v/v).

2.2. Apparatus

LC-MS/MS analyses were carried out in an Agilent Technologies 1200 series LC coupled to a 6420 triple quadrupole mass spectrometer with an electrospray ionization source (ESI) (Waldbronn, Germany). Chromatographic separation was carried out on a Synergi Hydro-RP 80A column (150 x 4.60 mm, 4 μm) from Phenomenex (Torrance, CA, USA). For the synthesis of SUPRAS, a Reax Heidolph vortex mixer (Schwabach, Germany) and an MPW -350R centrifuge from MPW Med- Instruments (Warchaw, Poland) were used. A sample evaporator/concentrator (SBHCONC/1 and SBH130D/3, Stuart, France) was used for evaporation of the SUPRAS extracts. SUPRAS volume was determined by measuring the height of the SUPRAS in Eppendorf tubes by using a digital calliper from Medid Precision, S.A. (Barcelona, Spain) and applying the formula for the volume of a cylinder.

[15] 2002/657/EC: Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. https://publications.europa.eu/en/publication-detail/-/publication/ed928116-a955-4a84-b10a-cf7a82bad858/language-en /Accessed 12 October 2018.

2.3. Microextraction/cleanup of benzimidazoles in milk by hexanol-based SUPRAS

Hexanol (0.1 mL) and THF (0.9 mL) were added to 1 mL of milk in 2-mL Eppendorf tubes. The mixture was vortex-shaken for 5 minutes at 2300 rpm and centrifuged at 21125g for 30 minutes at 20 ºC. The SUPRAS spontaneously formed through self-assembly and coacervation of hexanol and separated as a liquid phase (~ 470 µL) at the top of the solution that was easily separated and collected into an Eppendorf tube by means of a pipette. An aliquot of the SUPRAS (75 µL) was evaporated to dryness under a nitrogen stream at 60 ºC. The residue containing the precipitate of lipids was treated with 37.5 µL of a McIlvane buffer at pH 7.2 containing 10% MeOH (v/v) and 37.5 µL of acetonitrile and shaken for 1 min by hand for dissolving BDZ. Then, the extract was injected into the LC-MS/MS system for analysis (Figure 1).

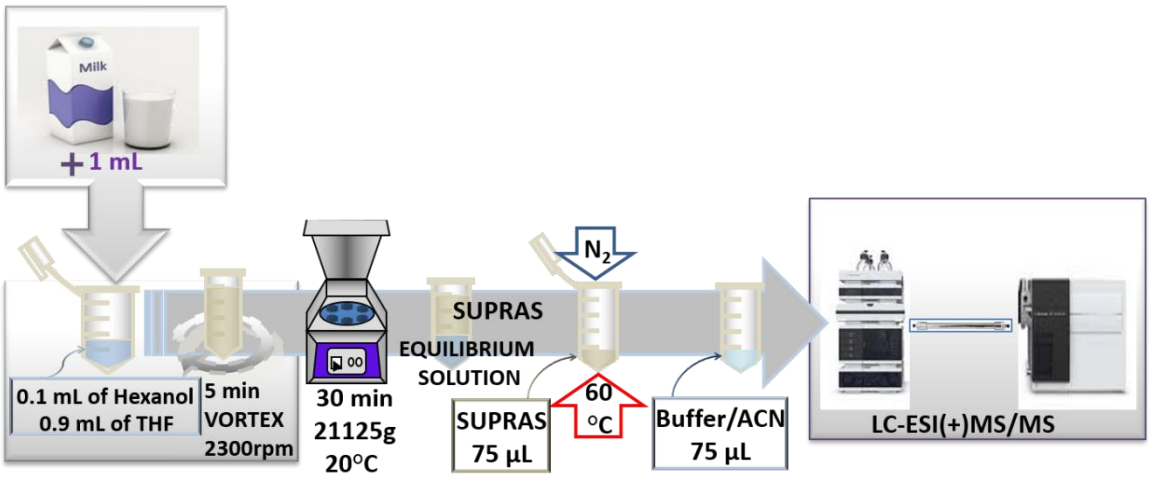


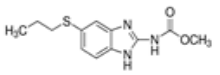
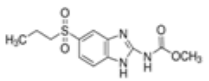
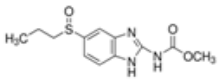
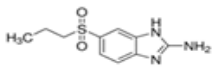
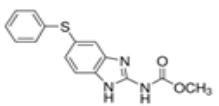
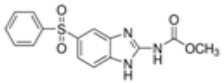
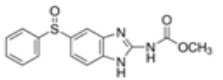
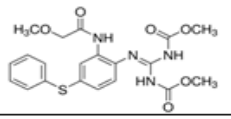
Figure 1. Schematic representation of the RAM-VOL-SUPRAS method prior to LC-MS/MS analysis.

2.4. LC-MS/MS analysis

The determination of the BDZ studied was undertaken by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The mobile phase consisted of ammonium formate buffer (2 mM; pH 7.5; solvent A) and acetonitrile (solvent B). The elution program started at 10 % of B, increased to 90 % for 12 min, and then was kept constant for 0.90 min. Finally, the system was re-equilibrated by establishing initial conditions for 6.70 min. The flow rate kept constant at 0.3 mL min^{-1} and the column temperature at 25ºC. The injection volume was set at 10 µL. MS/MS parameters were optimized by direct infusion and the most

abundant collision-induced fragments and the ion ratios were considered for quantification, while the second abundant fragments were used as qualifiers, as stated by the Commission Decision 2002/657/EC [15] (see Table 2). ESI source parameters were as follows: source gas: 50 psi of N_2 at 350 °C and capillary voltage: +4000V. Calibration curves were obtained by running calibration solutions prepared daily in McIlvane buffer at pH 7.2, containing 10% MeOH (v/v), at concentrations from the respective quantification limits for each analyte up to 500 μg L^{-1} (n=11, 8 replicates for each point). Peak-areas as function of concentrations were plotted for constructing calibration curves, and least-square regression was applied for calculating the concentration response correlation.

Table 2. Molecular structures, chemical properties and MS parameters used for the quantification of albendazole and fenbendazole families.

Compound name		Molecular structure	[a]M_W	[a]log K_{ow}	[a]pKa		[a]Hydrogen Bond Donor/Acceptor	Precursor ion (m/z)	Product ions (m/z) and relative abundance	Fragmentor (V)	Collision energy (eV)	Retention time (min)
					Acid groups	Basic groups						
Albendazole family	**ABZ**		265.331	2.2-2.9	N: 5.5	N: 13.1	2 / 4	266	234 (100%) 191 (66%)	40	16 32	15.9
	ABZ-SO_2		297.329	0.9-1.0	N: 3.5	N: 11.2	2 / 5	298	159 (100%) 192 (52%)	40	47 32	12.6
	ABZ-SO		281.33	0.8-0.9	N: 5.7	N: 13.2	2 / 5	282	159 (100%) 155 (32%)	55	20 32	11.9
	ABZ-NH_2-SO_2		239.073	0.7-0.75	N: 6.0	N: 13.3	2 / 4	240	133 (100%) 163 (9%)	50	28 12	11.7
Fenbendazole family	**FBZ**		299.073	3.1-4.0	N: 5.1	N: 12.7	2 / 4	300	159 (100%) 268 (99%)	40	36 16	16.5
	FBZ-SO_2		334.364	2.1-3.3	N: 3.4	N: 11.1	2 / 5	332	159 (100%) 300 (89%)	45	36 17	13.5
	OFZ		315.347	1.9-2.1	N: 4.1	N: 11.8	2 / 5	316	159 (100%) 191 (36%)	50	33 16	12.8
	FBT		446.478	[b]3.8	N: 5.8	-	3 / 7	447	383 (100%) 268 (6%)	40	12 28	17.3

[a] *Values calculated by Danaher et.al (2007).*

[b] *https://pubchem.ncbi.nlm.nih.gov/ (last access 31 October 2018).*

2.5. Samples

Samples whole milk (n=2, fat content: 3.6%), low-fat milk (n=2, fat content: 1.6%), and skimmed milk (n=2, fat content: 0.3 %) were purchased at local supermarkets. They were kept in the original package at 4 °C until use. For SUPRAS characterization, a low-fat milk sample was employed.

2.6. Method validation

The whole method was fully validated in low-fat milk following the Commission Decision 2002/657 [15]. Decision limits (CC_α) and detection capabilities (CC_β) were calculated by following the Commission Regulation (EU) No 37/2010 [4] and using the formulas described by Verdon [16].Method detection (MDL) and quantification (MQL) limits were calculated from blank samples fortified at concentrations close to the MQL of each compound, by using a signal-to-noise ratio of 3 and 10, respectively. Matrix effects were evaluated by spiking SUPRAS extracts (n=3), obtained from the treatment of low-fat milk, with the target analytes at 5 ng mL^{-1} each, and subjecting the extracts at evaporation and reconstitution according the procedure above (section about microextraction).. Signal suppression or enhancement (SSE, %) was calculated taking as reference the values obtained for standards in McIlvane buffer at pH 7.2, containing 10% MeOH (v/v), according to the procedure established by Matuszewski et al. [17].Values higher than 120% and lower than 70% were considered ion enhancement and suppression, respectively (Commission Decision 2002/657/EC) [15]. Because of the absence of commercially available certified reference materials (CRM), accuracy was evaluated in terms of recoveries by spiking low-fat milk at concentration of 0.5xMRL, 1xMRL and 1.5xMRL for each analyte (n=11). Precision, expressed as relative standard deviation (RSD, %), was evaluated in terms of repeatability and reproducibility by spiking six samples for three consecutive days at concentrations of 0.5xMRL, 1xMRL and 1.5xMRL for each analyte. The applicability of the method to whole, low-fat and skimmed milk was evaluated by analyzing both native and fortified samples at the MRLs. For this purpose, two different samples of each type of milk were analyzed by triplicate.

[16] E. Verdon, D. Hurtaud-Pessel, P. Sanders, Accredit. Qual. Assur., 11(2006) 58-62.
[17] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem., 75 (2003) 3019-3030.

3. RESULTS AND DISCUSSION

3.1. Phase diagram for hexanol-THF-milk ternary mixtures

The possibility of producing SUPRASs in milk meant for human consumption, which were able to simultaneously extract BDZs and remove matrix interferences, was here explored. For this purpose, the coacervation of hexanol in hydro-organic media was selected because of the high extraction efficiency and ability to remove proteins, carbohydrates and lipids of the SUPRASs produced [14]. The high water content of milk (~87.8%) [18], was expected to induce the coacervation of colloidal suspensions of hexanol in THF.

SUPRAS formation was investigated by adding different volume percentages of hexanol (from 0.1 to 10%) and of THF (from 10 to 80%) to milk samples, whose percentages can be deduced by difference, while keeping constant the total synthetic volume (i.e., 10 mL). Percentages of hexanol above 10% were not considered of analytical interest because the volume of SUPRAS formed increases with the content of amphiphile and, therefore, the concentration factors for analytes, defined as the V_{milk}/V_{SUPRAS} ratios, are expected to be very low. Figure 2A shows the binary phase diagram obtained for ternary mixtures of milk-hexanol-THF as a function of THF and hexanol concentration, expressed as volume percentage. For the purpose of comparison, this figure also includes the binary phase diagram obtained for water-hexanol-THF ternary mixtures (Figure 2B). SUPRAS formation in milk occurred in colloidal suspensions containing at least 1% of hexanol and 10% of THF and the SUPRAS region was much wider compared to that obtained in water (compare Figures 2A and 2B). This different behavior is a consequence of the influence of matrix components on SUPRAS formation [19]. In the case of milk, its higher specificity gravity (e.g., 1.028 – 1.033 kg L^{-1} at 15-20 °C) compared to that of water (e.g. 0.998-0.999 at 15-20 °C) resulted in an enhancement of SUPRAS immiscibility [14] and, accordingly, the region of SUPRAS formation became wider. In both cases, an isotropic solution was obtained when the THF content was increased above the boundaries found for SUPRAS formation.

[18] H. Hakk, N.W. Shappell, S.J. Lupton, W.L. Shelver, W. Fanaselle, D. Oryang, C.Y. Yeung, K. Hoelzer, Y. Ma, D. Gaalswyk, R. Pouillot, J.M. Van Doren, J. Agric. Food Chem., 64 (2016) 326-335.
[19] C. Caballo, M.D. Sicilia, S. Rubio, Supramolecular Solvents for Green Chemistry. In: F. Pena-Pereira, M. Tobiszewsky (eds) The Application of Green Solvents in Separation Processes. Elsevier, (2017).

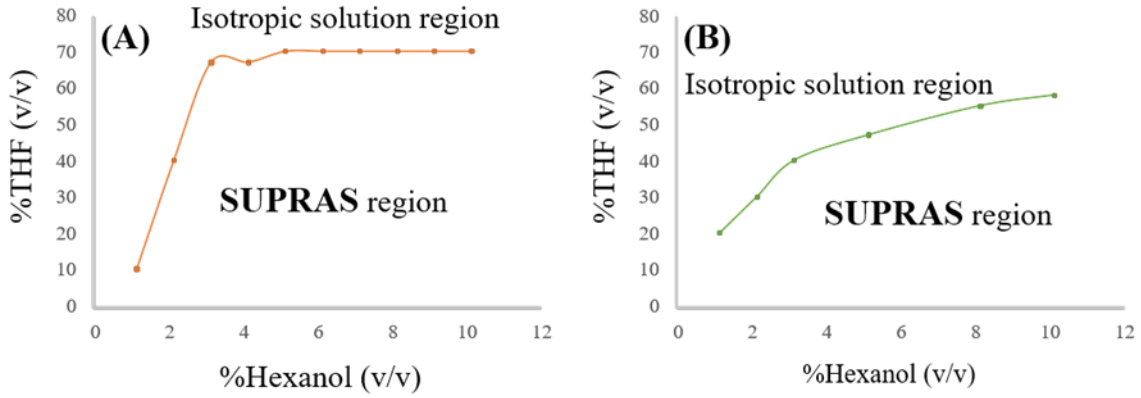


Figure 2. Phase diagrams for hexanol-based SUPRAS showing the region where a differentiated SUPRAS is formed (SUPRAS region) or not (Isotropic solution region) based on the proportions of THF, hexanol and milk (A) or water (B) in the synthetic solution. Region boundary proportions are represented (n= 3).

The volume of SUPRAS formed in milk was also checked. The study mainly focused on the SUPRAS region where higher concentration factors for BDZ are expected, that is, low percentages of hexanol and THF (i.e. hexanol 1-5% and THF 10-50%). It was noticed that the volume formed was consistent with the general prediction equation for RAM-VOL-SUPRAS volume (V_{SUPRAS}) proposed by Salatti et al. [14]:

$$V_{SUPRAS} = (10.7 \pm 0.3)\ Hexanol^{(0.0330 \pm 0.0007)\ THF}$$

SUPRAS volume increased linearly and exponentially with the volume percentages of hexanol and THF, respectively, within the intervals above specified. So, the highest concentration factors will be obtained at the lowest percentages of hexanol and THF. This equation allows us to set up the enrichment factors (defined as the ratio of analyte concentration in the SUPRAS to the initial concentration in the milk) to be achieved.

As for other alkanols [13], SUPRAS composition depended on the hydro-organic medium (i.e. milk/THF ratio) in which it formed. Thus, as the proportion of THF in the synthesis solution increased, the proportion of water and THF in the composition of the SUPRAS did. The higher content of water in the SUPRAS resulted in an increase of the size of aqueous cavities, as previously reported [14], which opens the possibility of using this SUPRAS as restricted access material for macromolecules.

3.2. Optimization of SUPRAS-based benzimidazole microextraction and sample cleanup

A good knowledge of the possible mechanisms of interactions between solvent and solutes is a requisite to establish effective extraction methodologies. Molecular structures and physico-chemical characteristics of BDZ are shown in Table 2. BDZ present acid and basic groups (pKa of the acid nitrogen in the range of 3.4–6.0, pKa of the basic nitrogen in the range of 11.1–13.3) [3], log K_{ow} in the range of 0.7–3.8, and contain aromatic rings and hydrogen bond donors/acceptors in their structure [1]. The hexanol-based SUPRAS are capable of solubilizing solutes with the following mixed-mode mechanism: polar and hydrogen bond donor/acceptor interactions with alcohol groups and dispersion interactions with the hydrocarbon chains [14, 20].

Aiming to optimize BDZ extraction and sample cleanup, SUPRAS synthetic conditions were evaluated since, as specified in the section above, hexanol-based SUPRAS are environment responsive and both their composition and vacuole sizes depend on hexanol/THF/matrix ratios in the synthetic solution. Optimization studies were restricted to the region of analytical interest, i.e. to volume percentages of hexanol and THF in the intervals 3-5% and 30-50%, respectively. No enough SUPRAS volume (< 75 μL) for LC-MS/MS analysis of the extract was obtained at lower volume percentages of both ingredients, considering a total volume for the synthetic solution of 2 mL (see Materials and methods section). On the other hand, the use of higher volume percentages was avoided in order to get the best possible concentration factors.

The suitability of the proposed methodology for removal of matrix interferences was evaluated by spiking SUPRAS extracts, obtained from the extraction of blank low-fat milk, with BDZ (5 ng mL-1), and subjecting them to evaporation and reconstitution as reported in Materials and methods. Selectivity alues, calculated as the percentage of signal suppression or enhancement (SSE, %) are shown in Table 3. They all were in the interval 80-115%, which confirmed the great capability of the hexanol-based SUPRAS for eliminating matrix effects in milk. It can be inferred from these results that proteins were effectively precipitated, independently of the SUPRAS composition tested. In fact, a white solid, compatible with protein precipitation, was always observed under any investigated conditions [14]. On the other hand, the size of the vacuoles of the reversed hexagonal aggregates of the SUPRAS, expected to increase as the THF in the synthetic solution does, was not important for

[20] A. Ballesteros-Gómez, L. Lunar, M.D. Sicilia, S. Rubio, Chromatographia (2018), In press, doi: 10.1007/s10337-018-3614-1.

removal of polysaccharides in milk. In fact, the carbohydrate content in milk is predominately lactose (a disaccharide) with trace amounts of monosaccharides and oligosaccharides. Therefore, the removal of oligosaccharides in this matrix was not important for achieving selectivity. Finally, the removal of lipids in the residue remaining after hexanol evaporation was also effective according the results obtained in Table 3.

Recoveries for BDZ as a function of the solvent set for residue reconstitution after hexanol evaporation was also investigated by spiking SUPRAS extracts, obtained from the extraction of low-fat blank milk, with BDZ (5 ng mL^{-1}). It was checked that re-extraction of analytes from the residue with the McIlvane buffer (pH 7.2) containing 10% MeOH (v/v) was very poor (e.g. 5.1±0.2, 108±11, 57±22, 17.2±0.2, 36±1, 14.3±0.7, 29±3 and 3.7±0.35.1 for ABZ, ABZ-SO_2, ABZ-SO, ABZ-NH_2-SO_2, FBZ, FBZ-SO_2, OFZ and FBT, respectively). The addition of acetonitrile to this buffer in a ratio 1:1 (v/v) increased the elution power of the reconstitution solution and quantitative recoveries were obtained for all BDZ while precipitated matrix lipids were left behind. The influence of the volumes of SUPRAS extract and reconstitution solution on BDZ recoveries is shown in Table 4. Quantitative recoveries for BDZ and a quicker evaporation of SUPRAS extracts were achieved for evaporation of 75 μL of SUPRAS and re-dissolution in 75 μL of Buffer/ACN. The combination of 150 and 75 μL resulted in poorer recoveries whilst 150/150 yielded no significantly different results when compared to 75/75 but duplicated the amount of time needed for evaporation. Thus, these conditions were selected and applied to further analyses.

Table 3. Selectivity, calculated as signal suppression or enhancement (SSE), for BDZ determination in low-fat milk as a function of SUPRAS synthetic conditions.

Synthetic conditions			Albendazole family SSE(%)±SD				Fenbendazole family SSE(%)±SD			
H (%)	THF (%)	Milk (%)	ABZ	ABZ-SO_2	ABZ-SO	ABZ-NH_2-SO_2	FBZ	FBZ-SO_2	OFZ	FBT
3	30	67	101±9	95±9	100±9	89±10	114±6	84±6	100±13	91±3
3	35	62	111±3	115±13	83±9	81±1	106±2	91±2	100±3	107±4
3	40	57	99±3	107±14	84±11	115±7	102±7	82±6	96±1	99±10
4	30	66	99±8	101±13	103±13	111±4	96±6	83±1	97±3	106±13
4	35	61	94±4	95±8	91±8	99±2	113±7	97±2	83±5	98±2
4	40	56	94±5	115±2	81±1	95±1	86±8	102±10	110±13	98±1
5	30	65	102±3	105±8	86±6	99±1	85±5	104±2	102±9	93±2
5	35	60	101±1	112±12	96±10	99±7	90±8	101±1	93±5	95±3
5	40	55	93±1	106±1	99±1	95±1	110±1	103±1	96±1	90±1
5	45	50	88±2	99±2	112±3	100±4	101±5	98±2	94±2	86±2
5	50	45	80±4	80±4	109±6	101±6	90±6	80±2	90±4	88±1

H= hexanol.
SUPRAS extracts spiked with the target analytes at 5 ng mL^{-1}.
SD= standard deviation (n=3).

Table 4. Recoveries obtained from different volumes of evaporated SUPRAS and reconstitution solution in the determination of anthelmintic benzimidazoles by LC-MS/MS prior to SUPRAS extraction.

SUPRAS extract (µL)	Reconstitution solvent (µL)	Albendazole family R(%)±SD				Fenbendazole family R(%)±SD			
		ABZ	ABZ-SO_2	ABZ-SO	ABZ-NH_2-SO_2	FBZ	FBZ-SO_2	OFZ	FBT
75	75	81±3	108±8	115±14	99±4	84±2	90±4	103±2	96±12
75	150	82±4	90±4	115±6	100±4	90±6	83±4	105±5	87±4
150	150	111±4	99±10	98±17	99±1	83±2	103±5	119±17	96±14
150	75	51±3	54±5	90±10	43±1	45±1	84±3	51±4	57±5

SUPRAS synthetic composition: 5 % of hexanol; 45 % of THF.
SUPRAS extracts spiked with the target analytes at 5 ng mL^{-1}.
R(%)= percentage of recovery.
SD= standard deviation (n=3).

Extraction efficiency for BDZ in milk as a function of the type of SUPRAS was investigated by spiking low-fat milk samples at a concentration of 2.5 ng mL^{-1} before SUPRAS extraction. Recoveries values in the range of 70-120% were considered quantitative (Commission Decision 2002/657/EC) [15]. Table 5 shows the results obtained. Overall, recoveries for BDZ progressively increased as the percentage of hexanol or THF in the solution did. Quantitative recoveries were obtained for the targeted BDZ, except for ABZ-NH_2-SO_2 (~ 45%), with an initial solution containing 5% of hexanol, 45% of THF and 50% of milk. Low absolute recoveries for ABZ-NH_2-SO_2 have been previously reported by extraction with solvents such ethyl acetate; e.g. 25% [21] and 47% [22], acetonitrile/dichloromethane [23] (77%) or even water [24] (not recovered) due to its higher polarity due to its higher polarity when compared to any other BDZ (see Table 2). Recoveries for ABZ-NH_2-SO_2 shown in Table 1 are mostly relative recoveries because the standard addition method was used for quantification.

A SUPRAS volume of 472 μL was produced under the optimal experimental conditions set for extraction of BDZ, consistent with the theoretical equation previously obtained for hexanol-based SUPRAS (see equation for V_{SUPRAS} specified above). With the aim to maximize yields for BDZ extraction, shaking time was checked in a range from 1 to 15 minutes. No major differences were found for recoveries and an intermediate time of 5 minutes was set. An optimum phase separation was totally achieved by 30 minutes of centrifugation (21125 g).

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Table 5. Recoveries for different SUPRAS initial conditions in the determination of anthelmintic benzimidazoles in low-fat milk by LC-MS/MS.

Synthetic conditions			V_{SUPRAS} (μL)	Albendazole family R(%)±SD				Fenbendazole family R(%)±SD			
H (%)	THF (%)	Milk (%)		ABZ	ABZ-SO_2	ABZ-SO	ABZ-NH_2-SO_2	FBZ	FBZ-SO_2	OFZ	FBT
3	30	67	173	48±8	22±4	43±7	19±3	34±6	49±8	35±6	67±12
3	35	62	204	41±3	29±2	55±4	16±1	29±2	38±3	29±2	63±5
3	40	57	240	34±5	28±4	53±8	20±3	26±4	36±5	29±4	68±10
4	30	66	230	52±10	30±6	62±12	25±5	38±7	43±8	40±8	79±15
4	35	61	272	55±3	41±2	71±4	27±2	36±2	61±4	57±3	70±4
4	40	56	320	56±10	40±7	69±12	35±6	44±8	67±11	50±9	82±14
5	30	65	288	60±6	37±4	59±6	33±4	47±5	55±6	62±7	69±7
5	35	60	340	63±7	47±5	73±8	39±4	43±5	67±8	63±7	65±8
5	40	55	401	52±1	77±1	84±1	52±1	50±1	63±1	67±1	88±1
5	**45**	**50**	**472**	**82±4**	**90±4**	**115±6**	**45±4**	**90±4**	**83±4**	**105±5**	**87±4**
5	50	45	557	62±4	110±8	95±7	46±6	68±5	69±5	95±7	69±5

H= hexanol.
R(%)= Recovery as percentage. Milk spiked at 2.5 ng mL^{-1}.
SD= standard deviation (n=3).
In bold, RAM-VOL-SUPRAS selected for further studies.
Total volume of synthetic solution: 2 mL.

3.3. Method validation

3.3.1. Linearity and sensitivity

Instrumental linear calibration curves were obtained for BDZ for concentrations from 0.33-0.59 to 500 μg L^{-1} (Table 6). Correlation coefficients ranged from 0.987 to 0.9996, confirming good fits for all BDZ. Decision limits (CC_α) and detection capabilities (CC_β) were evaluated following the current legislation (Commission Decision 2002/657/EC) [15], considering MRLs of 25 μg L^{-1} for each ABZ compound and 2.5 μg L^{-1} for each FBZ compound. CC_α values of 30.0, 28.2, 28.4, 27.7, 2.6, 2.7, 2.8 and 3.0 μg L^{-1}, and CC_β values of 34.8, 31.3, 31.7, 30.5, 2.8, 3.0, 3.1 and 3.5 μg L^{-1} for ABZ, ABZ-SO_2, ABZ-SO, ABZ-NH_2-SO_2, FBZ, FBZ-SO_2, OFZ, and FBT, respectively were obtained. Method detection (MDL) and quantification (MQL) limits for each analyte are also reported in Table 6. It should be highlighted that they are well below the legislated MRLs, i.e. 10 μg kg^{-1} for the fenbendazole family and 100 μg kg^{-1} for the albendazole family (Commission Regulation (EU) No 37/2010) [4].

Table 6. Linearity and sensitivity for the determination of anthelmintic benzimidazoles by RAM-VOL-SUPRAS extraction prior to LC-MS/MS

	Albendazole family				**Fenbendazole family**			
	ABZ	**ABZ-SO_2**	**ABZ-SO**	**ABZ-NH_2-SO_2**	**FBZ**	**FBZ-SO_2**	**OFZ**	**FBT**
[a]Linear range (μg/L)	0.35-500	0.51-500	0.46-500	0.59-500	0.33-500	0.43-500	0.40-500	0.36-500
Slope± SD	190±7	46±2	31.1±0.4	66±1	174±3	64±1	97±5	446±15
[b]r	0.997	0.997	0.9995	0.9992	0.9996	0.987	0.990	0.997
[c]LOD (μg/L)	0.11	0.22	0.21	0.27	0.11	0.12	0.11	0.11
[d] MDL (μg/L)	0.03	0.12	0.13	0.14	0.03	0.03	0.03	0.03
[e] MQL (μg/L)	0.09	0.39	0.44	0.46	0.10	0.11	0.10	0.09

[a]Instrumental quantification limit calculated by using a signal-to-noise ratio of 10.
[b]Correlation coefficient.
[c]Instrumental detection limit calculated by using a signal-to-noise ratio of 3.
[d]Method detection limits calculated for a signal-to-noise ratio of 3.
[e]Method quantification limits calculated for a signal-to-noise ratio of 10.
SD: standard deviation.

3.3.2. Accuracy

Table 7 shows the recoveries obtained at MRL, 0.5xMRL and 1.5xMRL concentrations for all the analytes (considering 25 μg L^{-1} for each ABZ compound and 2.5 μg L^{-1} for each FBZ compound). Recoveries were quantitative for all analytes, apart from ABZ-NH_2-SO_2 that ranged from 44 to 47%. Since for this analyte recoveries are lower than the accepted limits, results for ABZ-NH_2-SO_2 in real samples were calculated as relative recoveries. (Commission Decision 2002/657/EC) [15].

3.3.3. Precision

Relative standard deviations for BDZ were in the interval 3-13% at the MRLs, 7-19 % at 0.5xMRL, and 3-13% at 1.5xMRL values (Table 8). These intraday and inter-day precision values were below the recommended limits (i.e., <20% in the range 10-100 μg kg^{-1}) (Commission Decision 2002/657/EC) [15].

Table 7. Absolute recoveries obtained for ABZ and FBZ families for the determination of BZD by RAM-VOL-SUPRAS extraction prior to LC-MS/MS.

Compounds		Recovery (%)±SD		
		0.5xMRL[a]	MRL[b]	1.5xMRL[c]
Albendazole family	ABZ	84±12	92±11	96±12
	ABZ-SO_2	108±11	104±9	108±6
	ABZ-SO	100±8	103±7	109±6
	ABZ-NH_2-SO_2	44±4	45±3	47±2
Fenbendazole family	FBZ	86±6	79±2	84±2
	FBZ-SO_2	80±5	96±7	93±6
	OFZ	81±7	110±8	105±4
	FBT	81±6	83±10	98±12

[a] *0.5 times the Maximum residue level allowed in milk (ABZ family: 12.5 μg L^{-1} each, FBZ family: 1.25 μg L^{-1} each) n=11.*
[b] *Maximum residue level allowed in milk (ABZ family: 25 μg L^{-1} each, FBZ family: 2.5 μg L^{-1} each) n=11.*
[c] *1.5 times the Maximum residue level allowed in milk (ABZ family: 37.5 μg L^{-1} each, FBZ family: 3.75 μg L^{-1} each) n=11.*

Table 8. Intraday and inter-day precisions for the determination of anthelmintic benzimidazoles by RAM-VOL-SUPRAS extraction prior to LC-MS/MS.

Compounds		RSD Intraday (%)			RSD Interday (%)		
		0.5x MRL[a]	MRL[b]	1.5x MRL[c]	0.5x MRL[a]	MRL[b]	1.5x MRL[c]
ABZ family	ABZ	17	12	12	19	13	13
	ABZ-SO_2	10	8	7	11	9	10
	ABZ-SO	8	8	7	8	8	8
	ABZ-NH_2-SO_2	18	7	5	19	11	8
FBZ family	FBZ	8	3	3	7	8	7
	FBZ-SO_2	10	7	7	12	13	12
	OFZ	9	7	3	9	8	7
	FBT	13	12	12	14	12	12

[a] *0.5 times the Maximum residue level allowed in milk (ABZ family: 12.5 μg L^{-1} each, FBZ family: 1.25 μg L^{-1} each) n=11.*
[b] *Maximum residue level allowed in milk (ABZ family: 25 μg L^{-1} each, FBZ family: 2.5 μg L^{-1} each) n=11.*
[c] *1.5 times the Maximum residue level allowed in milk (ABZ family: 37.5 μg L^{-1} each, FBZ family: 3.75 μg L^{-1} each) n=11.*

3.4. Determination of benzimidazole in cow milk samples

The methodology developed was applied to six cow milk samples, which were sold for human consumption: whole (A-B), low-fat (C-D) and skimmed (E-F) milk. Both native and fortified samples, at the respective MRLs (ABZ family: 12.5 μg L^{-1} each, FBZ family: 1.25 μg L^{-1} each), were analyzed. Positive samples containing ABZ, FBZ-SO_2, OFZ and FBT in supermarket-sold cow milks were found. The ratio of the quantifier to the qualifier transition (see Table 2) was calculated for each unknown sample and it was compared to the ion ratio of the respective standards. The tolerance level defined by the European Commission Decision 2002/657/EC was considered for compound identification. Concentrations were 0.11-0.37 μg L^{-1}, and 0.25-2.55 μg L^{-1}, expressed as sum for ABZ and FBZ families, respectively. (Table 9 and Figure 3). All these values of BDZ residues in cow milk were below the legislated MRLs. (Commission Regulation (EU) No 37/2010) [4]. Quantitative recoveries (viz., 74-112%), obtained by spiking each sample at their respective MRLs, were obtained (triplicate analysis). ABZ-NH_2-SO_2 yields were calculated as relative recoveries. These results show the great applicability of the method, regardless of the milk fat content.

In summary, RAM-VOL-SUPRAS were employed for the hitherto unexplored quick, simple and successful determination of eight benzimidazole drugs in milk for human consumption, obtaining limits much lower than the legislated MRLs and improving those obtained by other sample treatments (viz. PPT, SPE, QuEChERS method and PMME, see Table 1). Satisfactory recoveries were obtained with the proposed method, as it was found for the other sample treatments mentioned, with the only exception of ABZ- NH_2- SO_2. However, the SUPRAS-based sample treatment here proposed has allowed to circumvent the tedious and sample/solvent-consuming pre-manipulation of milk using a lower volume of organic solvent per sample (i.e. 0.9 mL of THF) compared to that required by SPE[8,10,] (e.g. 5-11 mL) or QuEChERS[9] (e.g. 10 mL). Nevertheless, the most outstanding advantage achieved was the complete removal of interferences, which allowed the use of external calibration, and consequently, a considerable saving of time and cost. Furthermore, the method has been validated as an excellent way for removing matrix effect prior to LC-MS/MS analysis, regardless of the milk fat content. We hypothesize that this methodology, that spreads for the first time the applicability of SUPRAS to milk, could be extended to the treatment of other foodstuff samples with high amounts of fat.

Table 9. Determination of BDZ in cow milk samples by the RAM-VOL-SUPRAS extraction prior to LC-MS/MS methodology.

Samples	[a]Found concentration±SD (μg L^{-1}) - ([b]Recovery(%)±SD)								[d]Σ Albendazole group (μg L^{-1})	[e]Σ Fenbendazole group (μg L^{-1})
	Albendazole group				Fenbendazole group					
	ABZ	ABZ-SO_2	ABZ-SO	[c]ABZ-NH_2SO_2	FBZ	FBZ-SO_2	OFZ	FBT		
Whole Milk A	0.37±0.04 (99±10)	ND (105±6)	ND (97±8)	ND (84±5)	ND (81±1)	1.05±0.04 (92±4)	0.89±0.04 (100±5)	0.17±0.01 (89±4)	0.37	2.11
Whole Milk B	0.11±0.01 (98±11)	ND (109±8)	ND (97±8)	ND (87±10)	ND (81±3)	1.21±0.06 (92±6)	1.18±0.06 (100±6)	0.163±0.002 (88±6)	0.11	2.55
Low-fat milk C	<MQL (95±11)	ND (108±8)	ND (100±7)	ND (94±6)	ND (82±4)	0.59±0.03 (95±5)	ND (103±8)	0.136±0.002 (84±12)	<MQL	0.73
Low-fat milk D	0.27±0.03 (96±11)	ND (110±7)	ND (102±7)	ND (106±10)	ND (83±3)	ND (95±4)	0.25±0.02 (105±5)	ND (87±6)	0.27	0.25
Skimmed milk E	0.15±0.02 (98±9)	ND (112±5)	ND (106±8)	ND (106±2)	ND (87±3)	0.43±0.02 (101±5)	1.83±0.09 (106±6)	0.28±0.01 (88±4)	0.15	2.54
Skimmed milk F	0.52±0.03 (106±10)	ND (107±4)	ND (102±8)	ND (100±9)	ND (85±3)	0.32±0.02 (99±6)	1.78±0.09 (105±7)	ND (89±12)	0.52	2.10

[a] Average for three independent determinations

[b] Milk samples fortified at MRL: 25 μg L^{-1} for each ABZ compound and 2.5 μg L^{-1} for each FBZ compound

[c] Relative recoveries are calculated as a function of trueness obtained for low-fat milk

[d] Sum of found concentrations for the albendazole family

[e] Sum of found concentrations for the fenbendazole family

SD: Standard deviation; n=3.

ND: not detected.

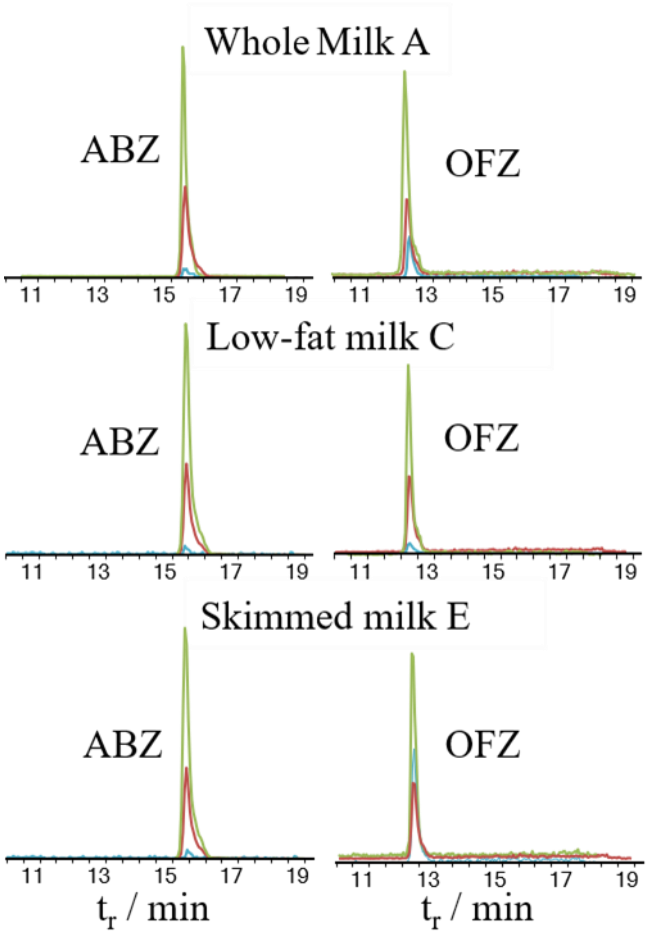

Figure 3. Extracted ion chromatograms obtained for ABZ (m/z 234) and OFZ (m/z 159) quantitation transitions from a calibration solution (red) at their respective MRLs (25 and 2.5 μg L^{-1}), a milk sample spiked before any treatment (green) at their respective MRLs (25 and 2.5 μg L^{-1}. Enrichment factor: 2.1), and an unspiked milk sample (blue, with found positive concentrations of 0.37 and 0.89 μg L^{-1} -whole milk A- and 0.15 and 1.83 μg L^{-1}-skimmed milk E- for ABZ and OFZ, respectively). HPLC conditions: ammonium formate buffer (2 mM; pH 7.5; solvent A) and acetonitrile (solvent B) from 0 min: 10 % B to12 min: 90% B.at 0.3 mL min^{-1}.Injection volume: 10 μL.

ABBREVIATIONS

BDZ, Benzimidazole drugs; EU, European Union; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PPT, protein precipitation; SPE, solid phase extraction; LPD, liquid phase deposition; QuEChERS, quick, easy, cheap, effective, rugged and safe extraction; PMME, polymer monolith microextraction; SUPRAS, supramolecular solvent; LC-IT-ToF/MS, liquid chromatography-ion trap-time-of-flight/mass spectrometry; HPLC-QQQ-MS, high performance liquid chromatography- triple quadrupole- mass spectrometry;

UHPLC-QQQ-MS, ultra-high performance liquid chromatography- triple quadrupole- mass spectrometry; PBS, phosphate buffer solution; EDTA, ethylenediaminetetraacetic acid; ACN, acetonitrile; THF, Tetrahydrofuran; CCβ, detection capability; LOQ, quantitation limit; MQL, method quantitation limit; ABZ, Albendazole; ABZ-SO2, Albendazole sulfone; ABZ-SO, Albendazole sulfoxide; ABZ-NH2-SO2, Albendazole-2-aminosulfone; FBZ, Fenbendazole; FBZ-SO2, Fenbendazole sulfone; OFZ, Oxfendazole; FBT, Febantel; MeOH, methanol; RAM-VOL-SUPRAS, restricted access volatile supramolecular solvent; RP, reverse phase; MW, molecular weight; pKa, log of acid dissociation constant; logKow, log of octanol-water partition coefficient; H, 1-hexanol; SSE, signal suppression/enhancement; MDL, method detection limit; MRL, maximum residue limit; CCα, decision limit; R, recovery.

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CONCLUSIONS

Conclusions.

General conclusions.

Part A: Determination of air and light sensitive endogenous antioxidants in serum by protein precipitation and high-performance liquid chromatography.

1. PPT (protein precipitation) by means of ACN coupled to liquid chromatography - UV detection has shown a great capability for the determination of endogenous antioxidants with high partition coefficients (i.e., XLogP3 13.5, 10.7 and 5.7 for β-carotene, α-tocopherol and retinol, respectively). Quantitative recoveries (viz. 70-120%), and good enough limits of detection (above 0.036 μg mL^{-1}) were also obtained thanks in part to the enrichment factor achieved (x2).

2. PPT with ACN has been proved to be a very simple and environmentally friendly serum treatment. Moreover, the ratio sample/ACN of 1:2 for inducing protein precipitation (>90%), the subsequent evaporation of the surnatant until dryness, which results in the absence of matrix effect, and the redissolution of the extract into mobile phase (ratio sample/reconstitution solution of 2:1) guaranteed low organic solvent employment, a positive enrichment factor (x2), and an optimum compatibility with the UV system.

3. The analytical method, which was employed in chapters 1-4, was fully validated following international guidelines. Suitable values for linearity, sensitivity, selectivity, precision and trueness were obtained.

Part B: SUPRAS-based matrix-independent platforms for quantifying multi compounds in biological matrices by LC-MS/MS for forensic, clinical and food quality control purposes.

1. Hexanol-based SUPRAS have successfully accomplished the extraction of analytes (i.e., amphetamines type stimulants and benzimidazole drugs) with very different physicochemical properties due to their ability to solubilize solutes in a mixed-mode mechanism (van der Waals, ionic, hydrogen bonding, polar and π- cation interactions). A great capability for cleaning-up several different types of biological

matrices (serum, urine, saliva, sweat, breast milk, cow milk, hair and finger nail) has been demonstrated, in all cases resulting on the removal of matrix interferents. Moreover, since SUPRAS are tailored solvents in which the amphiphile coacervation is triggered by the presence of water, they offer the possibility to be spontaneously and directly synthetized in liquid matrices and, prior hydrolysis, in solid samples. They act as restricted access material (RAM), or as volatile restricted access material (RAM-VOL-SUPRAS) when an evaporation step is applied for the elimination of phospholipids. In this way, small molecules are solubilized in the solvent, while interferences such proteins or polysaccharides are excluded by THF-based precipitation and/or formation of complexes, and size-exclusion phenomena, respectively. Hexanol-based SUPRAS allowed us to develop a platform for the determination of 5 ATS in seven different biological matrices for forensic applications, and to quantify 8 BDZ in cow milk regardless of fat content, by means of liquid chromatography coupled to tandem mass spectrometric detection (LC-MS/MS). The limits of the detection were below the cut-offs legislated and the maximum residue levels (MRLs) for ATS and BDZ, respectively.

2. An HFBA-based SUPRAS has been proposed by our group for the first time in this thesis as a new kind of alternative solvent for the extraction of very polar compounds (e.g., aminoacids and oligopeptides), which are not usually easily extracted by other solvents from biological matrices. The unique feature of this new SUPRAS is linked to the polar hydrophobicity shown by perfluorinated compounds. This SUPRAS was able to extract 20 aminoacids and 9 oligopeptides with negative log D values, achieving quantitative yields (recoveries >80%). Moreover, the incorporation of the amphiphile into the SUPRAS is 100%. Finally, the detection of the oligopeptide Opiorphin in saliva was successfully carried out by the direct injection of HFBA-based SUPRAS extracts in LC-MS/MS.

3. SUPRAS cleaning-up/extraction consists in a single treatment step, which guarantees an easy and effortless treatment with minimum time, sample and solvent consumption (e.g., time 15-35 min, sample ≤ 1 mL, organic solvent <2 mL). Therefore, this technique is in good agreement with the principles of green analytical chemistry.

4. The three analytical methods based on SUPRAS were fully validated following the parameters as requested by the respective international guidelines. Optimum values of linearity, sensitivity, selectivity, precision and trueness were achieved.

Specific conclusions.

Chapter 1: Blood serum retinol levels in Asinara white donkeys reflect albinism- induced metabolic adaptation to photoperiod at Mediterranean latitudes.

Protein precipitation for the simultaneously extraction and determination of β-carotene and retinol was successfully applied in serum of Asinara donkeys (Albino) and Sardo donkeys (Grey). In fact, the levels of these endogenous antioxidants determined by this method reflected the health state of the animals. Therefore, these antioxidants can be considered as markers of metabolic adaptation and photo-protection. Since the methodology involves just 0.3 mL of serum, it reached the objective of employing small amounts of sample for the analysis; this aspect is essential in methods in which a complicated sampling is required, such in cases where feral animals and air/light sensitive analytes are involved.

Chapter 2: Levels of LDH and CPK vary in blood serum of Asinara donkeys (albino) vs. Sardo 5 donkeys (pigmented) in presence of similar circulating α-tocopherol.

Taking advantage of the analytical results obtained when PPT treatment was applied to serum samples of feral animals, endogenous levels of α-tocopherol among two breeds of donkeys (eg., Asinara and Sardo) were successfully determined. For the first time, physiological ranges of α-tocopherol in donkeys were established. These results may be helpful for the diagnosis of adequate or deficient dietary supply, predisposing to pathologic conditions in donkeys.

Chapter 3: Supplementation of α-tocopherol/selenium in the diet of breeding stallions during negative photoperiod. Part I: effects on semen quality.

PPT treatment with ACN was successfully employed for determining α-tocopherol in serum of stallions. We were able to establish a correlation between the variation of serum antioxidant levels in these animals and semen quality, with the final aim of developing dedicated nutritional strategies useful to improve semen quality of breeding stallions.

Chapter 4: Baseline circulating levels of α-tocopherol in blood serum of feral Giara horses (*Equus ferus caballus* Linnaeus, 1758) and vitamin E status significantly vary with ALT from grazing to temporary captivity

Blood serum baseline levels of α-tocopherol in a group of Giara horses were investigated by employing of PPT with ACN and HPLC-UV analysis. Variations of circulating levels according to temporary captivity were detected. Baseline levels of α-tocopherol significantly decreased in horses after captivity (-32.5%), highlighting the direct effect of diet on circulating values after just four weeks of hay feeding.

Chapter 5: SUPRAS extraction approach for matrix-independent determination of amphetamine-type stimulants by LC-MS/MS.

A new analytical methodology has been developed for providing a truly universal, quickly, cheap, green and reliable sample treatment platform for monitoring the ATS expanding and startling abuse. The cleaning up/extraction of the 7 matrices was carried out by a SUPRAS made up of hexanol, tetrahydrofuran and sample, allowing the elimination of matrix effects due to the interferences contained in these complex matrices. Sample treatment was directly followed by LC/MS-MS analysis, which is considered the gold- standard in forensic field due its unique capabilities (i.e., reliability, sensibility and selectivity). The method was further applied for confirming the presence of ATS is 5 human urine samples which had resulted positive to immunoassay screening. The method here developed can be considered a green process since only low amounts of organic solvents were involved.

Chapter 6: Exploring polar hydrophobicity in organized media for extracting oligopeptides: application to the extraction of opiorphin in human saliva

A new kind of SUPRAS synthetized by the coacervation of HFBA in an acidic water solution was developed and characterized. Due to the presence of polar hydrophobicity due to C-F residues of the surfactant, this new solvent was able to quantitatively extract (viz. recoveries > 80%) very polar amino acids and oligopeptides with a log D value at pH 1- up to -3. Thus, very polar compounds, which usually are not easily extracted from complex matrices, can be obtained in extracts lacking matrix components, which could be useful for clinical and food

science applications. The methodology was further applied for quantifying the endogenous oligopeptide opiorphin in saliva by LC-MS/MS analysis.

Chapter 7: Restricted access volatile supramolecular solvents for single-step extraction/cleanup of benzimidazole anthelmintic drugs in milk prior to LC-MS/MS.

Volatile restricted access material Hexanol-based SUPRAS (VOL-RAM-SUPRAS) were efficiently applied for the extraction of 8 benzimidazole anthelminthic drugs in milk for human consumption. The method was proved to reduce the analysis time and to simplify the sample treatment (total time for sample treatment: 35 min) for the simultaneous determination of compounds with quite different physicochemical properties. The volatility of the SUPRAS allowed to obtain an excellent selectivity (SSE% above 100%), high sensibility (MDLs < legislated MRLs) and an excellent compatibility with LC-MS/MS system, by the introduction of a simple step of evaporation of the extract, which is subsequently redissolve, prior the injection. This methodology was applied to milks with different fat content and no differences in the achieved recoveries were found, confirming the restricted access capability of the SUPRAS. The method was further applied for the analysis of milks purchased in the local market. Residues of BDZ drugs below the legislated limits were detected.

ANNEXES

ANNEX I

Scientific publications arising from the Doctoral Thesis

Scientific publications arising from the Doctoral Thesis

1. **Blood serum retinol levels in Asinara white donkeys reflect albinism- induced metabolic adaptation to photoperiod at Mediterranean latitudes**

   M. G. Cappai, M. G. A. Lunesu, Francesca Accioni, M. Liscia, M. Pusceddu, L. Burrai, M. Nieddu, C. Dimauro, Gianpiero Boatto, W. Pinna

   Ecology and evolution 7 (2017) 390-398.

   Impact factor (Journal position/total number of Journals); Areas: Ecology, 2017(JCR): 2.34 (65/158, Q2)

2. **SUPRAS extraction approach for matrix-independent determination of amphetamine-type stimulants by LC-MS/MS**

   Francesca Accioni, D. García-Gómez, E. Girela, Soledad Rubio (2018)

   Talanta 182 (2018) 574-582.

   Impact factor (Journal position/total number of Journals); Area: Analytical Chemistry 2017(JCR): 4.244 (9/80, Q1)

3. **Levels of LDH and CPK vary in blood serum of Asinara donkeys (albino) vs. Sardo 5 donkeys (pigmented) in presence of similar circulating α-tocopherol**

   M.G. Cappai, Francesca Accioni, G. P. Biggio, R. Cherchi, Gianpiero Boatto, W. Pinna

   Submitted to The Veterinary Journal (July 2018)

   Impact factor (Journal position/total number of Journals); Areas: Agricultural and Biological Sciences; Animal Science and Zoology; Veterinary; Veterinary (miscellaneous) 2017(JCR): 1.773 (26/140, Q1)

4. **Baseline circulating levels of α-tocopherol in blood serum of feral Giara horses (*Equus ferus caballus* Linnaeus, 1758) and vitamin E status significantly vary with ALT from grazing to temporary captivity**

   Francesca Accioni, W. Pinna, F. Pudda, P. Wolf, Gianpiero Boatto, M.G. Cappai

   Submitted to Equine Veterinary Journal (Dicember 2018)

   Impact factor (Journal position/total number of Journals); Area: Veterinary Science 2017(JCR): 2.022 (17/140, Q1)

5. **Supplementation of α-tocopherol/selenium in the diet of breeding stallions during negative photoperiod. Part I: effects on semen quality**

   M.G. Cappai, A. Taras, I. Cossu, R. Cherchi, C. Dimauro, Francesca Accioni, Gianpiero Boatto, D. Gatta, W. Pinna

   Submitted to Animal Feed Science and Technology (July 2018)

   Impact factor (Journal position/total number of Journals); Area: Agriculture, Dairy & Animal Science 2017(JCR): 2.143 (6/60, Q1)

6. **Exploring polar hydrophobicity in organized media for extracting oligopeptides: application to the extraction of opiorphin in human saliva**

   Francesca Accioni, D. García-Gómez, Soledad Rubio

   To be submitted to Analytica Chimica Acta

   Impact factor (Journal position/total number of Journals); Area: Chemistry Analytical 2017(JCR): 5.123 (8/80, Q1)

7. **Restricted access volatile supramolecular solvents for single-step extraction/cleanup of benzimidazole anthelmintic drugs in milk prior to LC-MS/MS**

Francesca Accioni, N. Caballero-Casero, D. García-Gómez, Soledad Rubio
Journal of Agricultural and Food Chemistry. Publication Date (Web): December 5, 2018. **DOI:** 10.1021/acs.jafc.8b06003

Impact factor (Journal position/total number of Journals); Area: Agriculture, multidisciplinary, Chemistry, applied, Food Science & Technology 2017(JCR): 3.412 (2/57, Q1; 17/72, Q1; 18/133, Q1)