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A new method for the determination of benzophenone-UV filters in human serum samples by dispersive liquid–liquid microextraction with liquid chromatography–tandem mass spectrometry

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ABSTRACT

Benzophenone-UV filters (BP-UV filters) are extensively used in cosmetics products to avoid damaging effects of UV radiation. Despite their low toxicity, many research papers indicate that BP-UV filters are weak endocrine disruptors (EDCs). There are clear relationships between BP-UV filters exposure and several health disorders such as carcinogenesis and malformations observed in animals. In the present work, a new sample treatment procedure by dispersive liquid–liquid microextraction (DLLME) is proposed for the extraction of six BPs, namely benzophenone-1 (BP-1), benzophenone-2 (BP-2), benzophenone-3 (BP-3), benzophenone-6 (BP-6), benzophenone-8 (BP-8) and 4-hydroxybenzophenone (4-OH-BP), in human serum samples, followed by ultra-high performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) analysis. The method involves an enzymatic treatment to quantify the total content (free plus conjugated species) of BP-UV filters in serum. The extraction parameters were accurately optimized using multivariate optimization approach. Benzophenone-d_{10} (BP-d_{10}) was used as surrogate. Limits of quantification (LOQs) ranged from 0.4 to 0.9 ng mL^{-1} and inter-day precision (evaluated as relative standard deviation) ranged from 1.9% to 13.1%. The method was validated using matrix-matched calibration and a recovery assay. Recovery rates for spiked samples ranged from 97% to 106%, and acceptable linearity was obtained up to concentrations of 40 ng mL^{-1}. The method was applied to the determination of the target compounds in human serum samples from 20 randomly selected anonymous individuals.

1. Introduction

Harmful health effects of UV radiation have been demonstrated many years ago. The increasing exposure to UV irradiation raises a growing demand for chemicals which protect the skin against sunburn, photoageing, skin cancer, and photodermatosis. These chemicals, commonly referred as ultraviolet (UV) filters, are able to absorb UV solar radiation and protect human skin from direct exposure. UV filters posses single or multiple aromatic structures, commonly conjugated with different chemicals groups (carbonyl, double bounds, etc.), that are able to absorb UV radiation photons. These compounds are used extensively in sunscreens, cosmetic products such as facial day creams, after-shave products, makeup formulations, lipsticks, shampoos, and in plastic based packaging materials. In the European Union, 26 different organic compounds are permitted for those uses. The maximum content of these compounds in cosmetics is regulated by the actual legislations, at a usual concentration between 0.1% and 10% (w/w) [1].

There are 12 well-known BP-UV filters, namely benzophenone-1 (BP-1) to benzophenone-12 (BP-12), as well as other less usual compounds as 2-hydroxybenzophenone (2-OH-BP) or 4-hydroxybenzophenone (4-OH-BP). In cosmetics and personal care products, BP-1 and BP-3 are usually used in the formulation of nail polishes and enamels. These BP-UV filters are also used in the manufacturing of bath products, makeup products, hair products, sunscreens and skin care products. These compounds protect cosmetics and personal care products from deterioration by absorbing, reflecting, or scattering UV rays. When used as sunscreen ingredients, BP-3 and BP-4 protect the skin from UV rays.

There are increasing evidences that BP-UV filters are able to interfere with the endocrine system. In vitro studies have shown that BPs stimulate the proliferation of the breast cancer cell line MCF-7 due to their estrogenic activity, and that these compounds have antiandrogenic activity too [2,3]. These conclusions have correlation with the results of several in vivo research works. BP-2 is able to accumulate in fish and to induce a lot of abnormalities in

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many sexual functions and features [4]. Carcinogenesis and reproductive organ malformations were observed in rodents after exposure to BP-UV filters [5,6].

There are two main metabolic pathways of BP-UV filters. Biotransformation is usually divided into two main phases, commonly known as phases I and II. Phase I is usually an oxidative and/or hydrolytic process whereby lipophilic xenobiotics are turned into more polar species, and thus, more easily excretables. Next, in phase II, if the phase I metabolites or even the parent compound have not yet been excreted, they can react with highly polar species such as glucuronic acid, sulfate, methionine, cysteine or glutathione, resulting in the so-called conjugates [7]. BPs can suffer yet a "cross-transformation" to other types of BPs which often show more dangerous disrupting activities than the original forms [8]. It has been reported that BP-3 is metabolized to BP-1 and BP-8 in animals [8–11], and there are some evidences about BP-1 possesses higher estrogenic activity than BP-3 [2,10,12–14]. Others benzophenone derivatives such as BP-2 and 4-OH-BP are also metabolites of BP-3. In fact, the occurrence of these BP-3 metabolism products has been previously reported in human urine [15].

In the last years some methods for analysis of BP-UV filters in different environmental matrices have been proposed: waters [16–19], indoor dust [20], soils and sediments [21,22] have been the main types of studied samples. However, only a few methods have been reported for biological samples, being urine the most studied matrix [23–32]. To our knowledge, there is a lack of published analytical methods to assess human exposure to BP-UV filters in other kinds of samples: two methods in blood [26,31], two in serum [33,34], one in milk [35], one in semen [36] and one in placental tissue [37].

The use of the highly-potential microextraction techniques as the dispersive liquid–liquid microextraction (DLLME), developed by Rezaee and co-workers in 2006 [38], have provided very good results in complex samples. The basic principles of the DLLME have been explained elsewhere, as well as the advantages over the traditional extraction techniques and other microextraction techniques [38]. DLLME has been widely used in the analysis of many types of pollutants and organic compounds in environmental matrices, in food samples and in biological human samples [39,40]. However, DLLME has hardly been used in analysis of BP-UV filters in human samples.

Recently, Tarazona et al. [34] have proposed a method to determine BP-3 and its main metabolites in human serum by DLLME–LC–MS/MS. An acidic hydrolysis and protein precipitation with HCl 6 M (1:1) (100 °C, 1 h) were carried out before extraction. Acetone and chloroform were used as disperser and extraction solvents, respectively. However, the authors only determine the total content of target benzophenone-UV filters without analyzing the free form of them.

The aim of this work is to develop an accurate, selective and sensitive DLLME procedure followed by UPLC–MS/MS analytical method for the simultaneous determination of six BP-UV filters in human serum samples. The proposed method has been validated and satisfactorily applied for the determination of these compounds (free and total) in human serum samples from 20 randomly selected individuals.

2. Experimental

2.1. Chemical and reagents

All reagents were analytical grade unless otherwise specified. Water (18.2 MΩ cm) was purified using a Milli-Q system from Millipore (Bedford, MA, USA). BP-1 (2,4-dihydroxybenzophenone), BP-2 (2,2′,4,4′-tetrahydroxybenzophenone), BP-3 (2-hydroxy-4-methoxybenzophenone), BP-6 (2,2′-dihydroxy-4,4′-dimethoxybenzophenone), BP-8 (2,2′-dihydroxy-4-methoxybenzophenone), 4-OH-BP (4-hydroxybenzophenone) and BP-d_{10} were supplied by Sigma-Aldrich (Madrid, Spain). Stock standard solutions (100 mg L^{-1}) for each compound were prepared in methanol and stored at 4 °C in the dark. These solutions were stable for at least 4 months. Working standards were prepared by dilution with methanol immediately before use. 4-methylumbelliferyl glucuronide, 4-methylumbelliferyl sulfate, β-glucuronidase/sulfatase and *Helix pomatia* (H1) were purchased from Sigma-Aldrich (Madrid, Spain). $^{13}C_4$-4-methylumbelliferone was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). A mixture of $^{13}C_4$-4-methylumbelliferone, 4-methylumbelliferyl sulfate, and 4-methylumbelliferyl glucuronide was prepared in water and stored at 4 °C until use. The enzyme solution was prepared daily for each run by dissolving 6 mg of β-glucuronidase/sulfatase (3,000,000 U g solid $^{-1}$) in 1 mL of 1 M ammonium acetate/acetic acid buffer solution (pH 5.0). Methanol, ethanol, acetone and acetonitrile (HPLC-grade) were purchased from Merck (Darmstadt, Germany). LC–MS grade methanol and water, chlorobenzene (ClBz), trichloromethane (TCM), carbon tetrachloride (TCC) and ammonia (25%) were purchased from Sigma-Aldrich. Sodium chloride and ammonium acetate were supplied by Panreac (Barcelona, Spain). Fetal bovine serum was purchased from IBIAN TECHNOLOGIES (Zaragoza, Spain).

2.2. Instrumentation

UPLC–MS/MS analysis was performed using an ACQUITY UPLC™ H-Class (Waters, Manchester, UK), consisting of ACQUITY UPLC™ binary solvent manager and ACQUITY UPLC™ sample manager. A Xevo TQS tandem quadrupole mass spectrometer (Waters) equipped with an orthogonal Z-spray™ electrospray ionization (ESI) source was used for BPs detection.

All pH measurements were made with a Crison (Crison Instruments S.A., Barcelona, Spain) combined glass–Ag/AgCl (KCl 3 M) electrode using a previously calibrated Crison 2000 digital pH-meter. A thermo shaker (model MS-100, Optimum Ivymen System, Cornecta, Spain) was used for enzymatic treatment.

Statgraphics Plus version 5.0 (Manugistics Inc., Rockville, MD, USA, 2000) was used for statistical and regression analyses (linear mode).

2.3. Sample collection and storage

Human serum samples were collected from the 20 volunteers at the Management Clinical Laboratory Unit of the *San Agustín* Hospital (Linares, Jaén, Spain). Samples were anonymized, frozen at −86 °C and stored until analysis in our laboratory. All volunteers signed their informed consent to participate in the study.

2.4. Basic procedure

2.4.1. Enzymatic treatment

In order to evaluate free and total amounts of benzophenone-UV fiters in serum, each sample was treated in two different ways. One sample was processed without addition of enzymes and the other one was treated with β-glucuronidase/sulfatase. For experiments without enzymatic treatment, an aliquot of serum (1.0 mL) was added into a centrifuge glass tube and spiked with 10 μL of surrogate (BP-d_{10}) standard solution (20 μg L^{-1}). To analyze the total (free + conjugated) concentration of the tested benzophenone-UV fiters, 1.0 mL of sample was spiked with 10 μL of surrogate (BP-d_{10}) standard solution and 50 μL of enzyme solution (β-glucuronidase/sulfatase). Furthermore, 25 μL of 4-methylumbelliferyl glucuronide/4-methylumbelliferyl sulfate/$^{13}C_4$-4-methylumbelliferone standard mixture (4 μg mL^{-1}) were added to check the extent of

the deconjugation. After mixing, the sample was incubated at 37 °C for 24 h. 4-methylumbelliferyl sulfate and 4-methylumbelliferyl glucuronide were deconjugated to free 4-methylumbelliferone, and the 4-methylumbelliferone/$^{13}C_4$-4-methylumbelliferone peak area ratio was monitored to assess the correct activity of the enzyme. Deconjugation efficiencies were close to 100% in all cases.

2.4.2. Sample preparation

Prior to the DLLME procedure a serum protein removal was necessary. Acetone (1.0 mL) was added to 1.0 mL of human serum. The mixed solution was vortexed for 30 s and centrifuged for 10 min at 3000 rpm (1460*g*). The supernatant was transferred to the volumetric flask and diluted with 5% NaCl aqueous solution (w/v) to 10.0 mL. The pH was adjusted to 2.0 with 0.1 M HCl.

2.4.3. DLLME procedure

This solution was placed in a 15 mL screw-cap glass test tube. Next, 3.5 mL of acetone (disperser solvent) and 500 μL of TCM (extraction solvent) were mixed and injected rapidly into the aqueous sample with a syringe. The mixture was gently shaken for 10 s, and centrifuged for 20 min at 4000 rpm (2600*g*). All sedimented phase volume was transferred to a clean glass vial using a 1.0 mL micropipette. The organic phase was evaporated under a nitrogen stream. The residue was dissolved with 100 μL of a mixture consisting of methanol (0.1% ammonia)–water (0.1% ammonia), 60:40 (v/v), and then vortexed for 30 s.

2.4.4. Chromatographic conditions

Chromatographic separation of compounds was performed using an ACQUITY UPLC® BEH C_{18} (50 mm × 2.1 mm i.d., 1.7 μm particle size) from Waters (UK). Standards and samples were separated using a gradient mobile phase consisting of 0.1% (v/v) ammoniacal aqueous solution (solvent A) and 0.1% (v/v) ammonia in methanol (solvent B). Gradient conditions were as follows: 0.0–3.5 min, 60% B; 3.5–4.0 min, 60–100% B; 4.0–6.5 min, 100% B and back to 60% in 0.1 min. Flow rate was 0.25 mL min^{-1}. The injection volume was 10 μL. The column temperature was maintained at 40 °C. Total run time was 10.0 min.

2.4.5. Mass spectrometric conditions

ESI was performed in positive ion mode. The tandem mass spectrometer was operated in the selected reaction monitoring (SRM) mode and Q1 and Q3 quadrupoles were set at unit mass resolution. The mass spectrometric conditions were optimized for each compound by continuously infusing standard solutions (1 mg L^{-1}). The ion source temperature was maintained at 150 °C. Instrument parameters were as follows: capillary voltage, 0.60 kV; source temperature, 150 °C; desolvation temperature, 500 °C; cone gas flow, 150 L h^{-1}; desolvation gas flow, 500 L h^{-1}; collision gas flow, 0.15 mL min^{-1}, and nebulizer gas flow, 7.0 bar. Nitrogen (99.995%) was used as cone and desolvation gas, and argon (99.999%) was used as collision gas. Other adjustments such as collision energies (CE) and cone voltages (CV) were optimized for each analyte. Dwell time for each compound was set at 25 ms. Optimized parameters for each compound are listed together with the mass transitions in Table 1.

Table 1
Selected transitions and optimized mass spectrometry parameters for the analysis of benzophenone-UV fiters.

Compound	Transitions	CV (V)	CE (eV)
4-OH-BP	199.0 → 120.8[a]	36	20
	199.0 → 104.8[b]	36	18
BP-1	214.9 → 136.8[a]	2	18
	214.9 → 80.8[b]	2	32
BP-2	245.1 → 134.8[a]	40	16
	245.1 → 108.9[b]	40	22
BP-3	229.0 → 150.8[a]	4	20
	229.0 → 104.9[b]	4	18
BP-6	275.0 → 150.9[a]	14	18
	275.0 → 94.9[b]	14	34
BP-8	245.0 → 120.9[a]	14	20
	245.0 → 150.9[b]	14	20
BP-d_{10}	193.1 → 109.8[a]	18	16
	193.1 → 81.8[b]	18	30

CV, cone voltage. CE, collision energy.
[a] SRM transition used for quantification.
[b] SRM transition for confirmation.

3. Results and discussion

3.1. Optimization of DLLME conditions

3.1.1. Selection of disperser solvent and extraction solvent

The disperser–extractant solvent pair is one of the most important factors in DLLME optimization. In a first series of experiments, 1.0 mL aliquots of spiked human serum with 10 μg L^{-1} of all studied BPs were treated as described in Section 2.4.2. Mixtures of 1.0 mL of different disperser solvents (acetonitrile, methanol, ethanol and acetone) and 0.1 mL of extractant solvent (TCC, TCM and ClBz) were added to 10 mL of sample solution (three experimental replicates). The highest responses for all compounds corresponded to acetonitrile–TCM and acetone–TCM pairs (data not shown). Because of the lower price of acetone, acetone–TCM was selected as the optimum disperser–extractant pair.

3.1.2. Effects of the volume of extractant and dispersant, pH sample, salt addition and extraction time

The effects of sample pH, salt (NaCl) percentage, extraction time (defined as the period during which the sample is shaken after addition of the binary extraction mixture and before centrifugation), volume of extractant and volume of dispersant on the performance of the method were simultaneously investigated using a two-level 2^{5-1} fractional factorial design, with three replicates of the central point. In order to minimize the content of BPs that are naturally found in human serum samples, a pool with very low concentration of these compounds was spiked with 10 μg L^{-1} of all BPs and used in diagnostic and optimization studies. The residue (see Section 2.4.2) was dissolved with 100 μL of a BP-d_{10} solution (100 μg L^{-1}) prepared in methanol (0.1% ammonia)–water (0.1% ammonia), 60:40 (v/v). The response variable used in for that experiment was relative area. Experimental domain and standardized effects of factors are summarized in Table 2.

All variables have a significant influence (95% confidence level) on the extraction procedure. Influence of pH, NaCl percentage and extraction time are the same for all compounds and the higher responses were obtained with low pH, high NaCl percentage and low extraction time. Therefore, in order to simplify the optimization process, pH = 2.0, addition of a 5% of NaCl and 10 s of shaking time were selected.

3.1.3. Volume of extractant and dispersant

The optimal volume of acetone and TCM were evaluated with a Doehlert surface response design (three central point replicates). This experimental design allows the simultaneous optimization of

Table 2
Experimental domain and standardized effects of investigated factors on the performance of the method.

Factor	Level	
	Low	High
Acetone volume (mL)	0.5	3.5
TCM volume (μL)	150	750
Sample pH	2	6
NaCl (%)	0	5
Shaking time (s)	10	120

Factor	Standarized effect values					
	4-OH-BP	BP-1	BP-2	BP-3	BP-6	BP-8
Acetone volume (*A*)	0.098*	0.036*	0.004*	0.334*	0.098*	0.216*
TCM volume (*B*)	0.098*	0.029*	0.012*	0.209*	0.026	0.118*
pH (*C*)	0.022	−0.034*	−0.021*	−0.864*	−0.463*	−0.663*
% NaCl (*D*)	0.007	0.018*	0.012*	0.465*	0.301*	0.387*
Shaking time (*E*)	−0.141*	−0.042*	−0.002	−0.358*	−0.159*	−0.258*
AB	0.100*	0.021*	0.006*	0.122*	−0.052*	0.035
AC	0.094*	0.004	−0.001	0.047	0.088*	0.067
AD	−0.089*	−0.018*	−0.003*	−0.342*	−0.181*	−0.261*
AE	−0.044*	−0.002	−0.001	0.009	0.023	0.015
BC	0.086*	0.013*	−0.001*	−0.094	0.007	−0.041
BD	−0.098*	−0.016*	0.002*	−0.227*	−0.115*	−0.171*
BE	−0.044*	−0.002	0.003*	0.391*	0.241*	0.316*
CD	−0.121*	−0.034*	−0.016*	−0.536*	−0.261*	−0.401*
CE	−0.102*	−0.018*	0.001	−0.331*	−0.146*	−0.238*
DE	0.080*	0.016*	−0.003*	0.115*	0.096*	0.104*

* Statistically significant factors (95% confidence level).

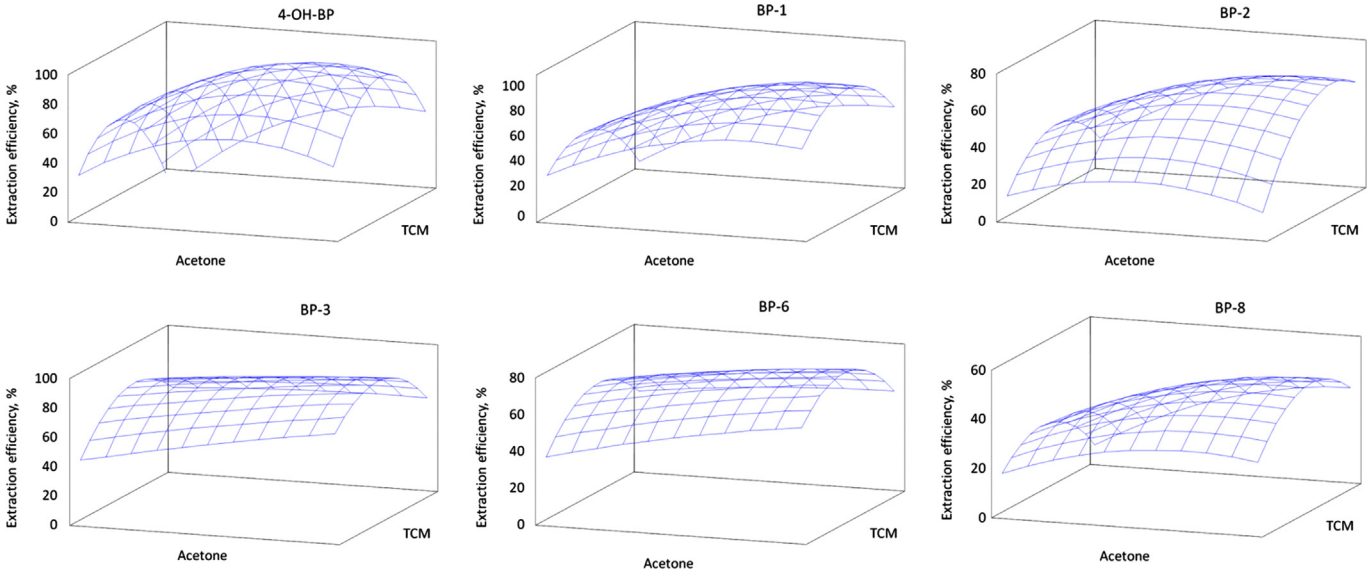

Fig. 1. Response surfaces obtained by the Doehlert design.

two variables, studying one of them at three levels (in this case the volume of acetone) and the second one at five levels (in this case the volume of TCM). Spiked human serum with 10 μg L^{-1} of all compounds was used in that experiment.

Response surfaces of studied BPs are given in Fig. 1. In all cases, optimal extraction efficiencies were obtained at maximal volume of acetone, 3.5 mL, and central volume of trichloromethane, 0.45 mL.

3.2. Analytical performance

Due to a lack of BPs free human serum samples, fetal bovine serum was used for calibration purposes. This strategy has been employed by several authors. In this way, calf serum or horse serum has been used for these purposes in the determination of different EDCs in human serum [35,41].

An eight concentration level calibration curve was built. Each level of concentration was made in triplicate. Calibration curves were constructed using analyte/surrogate peak area ratio versus concentration of analyte. Calibration graphs were made using SRM mode. BP-d_{10}, at a concentration of 20 μg L^{-1}, was used as surrogate.

In order to estimate the presence/absence of matrix effect, two calibration curves were obtained for each compound, one in distilled water and the other one in the fetal bovine serum. The Student's *t*-test was applied in order to compare the calibration

curves. First, the variances estimated as $S^2_{y/x}$ were compared by means of a Snedecor's *F*-test. The Student's *t*-test showed statistical differences among slope values for the calibration curves in all cases and consequently, the use of matrix-matched calibration was necessary. Table 3 shows the analytical parameters obtained.

3.3. Method validation

Validation in terms of linearity, sensitivity, accuracy (trueness and precision), and selectivity, was performed according to the US Food and Drugs Administration (FDA) guideline for Bioanalytical Method Validation [42].

3.3.1. Linearity

A concentration range for the minimal quantified amount (0.6 ng mL^{-1} for 4-OH-BP, BP-1 and BP-2; 0.7 ng mL^{-1} for BP-3; 0.9 ng mL^{-1} for BP-6 and 0.4 ng mL^{-1} for BP-8) to 40 ng mL^{-1} was selected. Linearity of the calibration graphs was tested using the determination coefficients (% R^2) and the *P*-values (% P_{lof}) of the *lack-of-fit* test [43]. The values obtained for R^2 ranged from 99.2% for BP-2 to 99.5% for BP-6, and P_{lof} values were higher than 5% in all cases. These facts indicate a good linearity within the stated ranges.

3.3.2. Limits of detection and quantification

Limit of detection (LOD) and limit of quantification (LOQ) are two fundamental parameters that need to be examined in the validation of any analytical method to determine if an analyte is present in the sample. The LOD is the minimum amount of analyte detectable in the sample, while the LOQ is the minimum amount that could be quantified. In this work, these parameters were calculated by taking into consideration the standard deviation of residual $S_{y/x}$, the slope b of the calibration curve and an estimate s_0 obtained by extrapolation of the standard deviation of the blank [44]. The LOD was $3s_0$ and the LOQ was $10s_0$. Limits of quantification ranging from 0.4 to 0.9 ng mL^{-1} were obtained. These results

Table 3
Analytical and statistical parameters of the proposed analytical method for the analysis of benzophenone-UV filters.

	4-OH-BP	BP-1	BP-2	BP-3	BP-6	BP-8
a	1.2×10^{-1}	2.4×10^{-2}	1.9×10^{-2}	2.1×10^{-1}	1.3×10^{-1}	4.9×10^{-2}
s_a	5.8×10^{-3}	4.2×10^{-3}	1.2×10^{-3}	1.3×10^{-2}	9.4×10^{-3}	6.2×10^{-3}
b (mL ng^{-1})	2.0×10^{-1}	1.2×10^{-1}	3.1×10^{-2}	3.8×10^{-1}	3.0×10^{-1}	2.0×10^{-1}
s_b (mL ng^{-1})	2.9×10^{-3}	2.1×10^{-3}	5.8×10^{-4}	6.3×10^{-3}	4.6×10^{-3}	3.1×10^{-3}
R^2 (%)	99.5	99.3	99.2	99.4	99.5	99.5
LOD (ng mL^{-1})	0.2	0.2	0.2	0.2	0.3	0.1
LOQ (ng mL^{-1})	0.6	0.6	0.6	0.7	0.9	0.4
LDR (ng mL^{-1})	0.6–40.0	0.6–40.0	0.6–40.0	0.7–40.0	0.9–40.0	0.4–40.0

a, Intercept; s_a, intercept standard deviation; *b*, slope; s_b, slope standard deviation; R^2, determination coefficient; LOD, limit of detection; LOQ, limit of quantification; LDR, linear dynamic range.

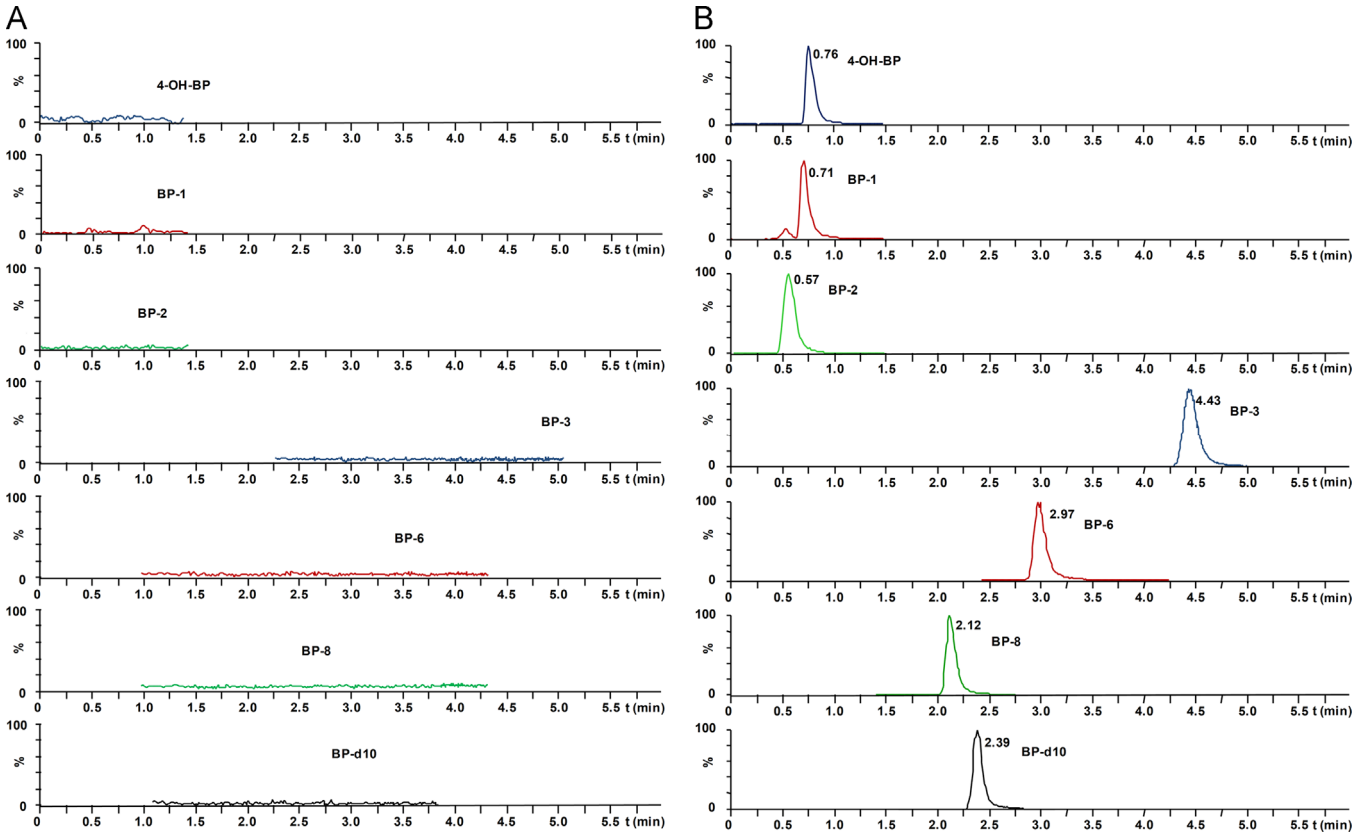

Fig. 2. SRM mode chromatograms: (A) a blank fetal bovine serum sample; and (B) a fetal bovine serum spiked sample (5 ng mL^{-1} of each studied analyte).

are summarized in Table 2. These values of LOQs demonstrate a better sensitivity of the proposed method in comparison with others previously proposed procedures for determination of benzophenones in human serum (1.7 ng mL^{-1} for BP-3 [33]; 22 ng mL^{-1} for BP-8, 27 ng mL^{-1} for BP-3 and BP-1 [34]).

3.3.3. Selectivity

The specificity of the method was demonstrated by comparing the chromatograms corresponding to the procedure blank and the obtained in fetal bovine blank serum. No interferences from endogenous substances were observed at the retention time of the analytes, and no peak BP was found in procedural blanks. These findings suggest that the spectrometric conditions ensured high selectivity of the UPLC–MS/MS method. The specificity of the method was demonstrated by analyzing of the chromatograms of the procedure blank and the corresponding fetal bovine blank serum.

SRM mode chromatograms of a blank fetal bovine serum (*A*) and a spiked fetal bovine serum –5 ng mL^{-1} of each studied analyte–(*B*) are shown in Fig. 2.

3.3.4. Accuracy (precision and trueness)

Due to the absence of certified materials, in order to evaluate the trueness and the reproducibility of the method, a study with spiked blank fetal bovine serum samples, at three concentrations levels for each compound (2, 20 and 40 ng mL^{-1}), was performed on six consecutive days. The precision was expressed as relative standard deviation, % RSD, and the trueness was evaluated by a recovery assay. The precision and the trueness of the proposed analytical method are shown in Table 4.

Trueness was evaluated by determining the recovery of known amounts of the tested compounds in blank fetal bovine serum samples. Samples were analyzed using the proposed method and the concentration of each compound was determined by interpolation in the standard calibration curve within the linear dynamic range and compared to the amount of analytes previously added to the samples. A recovery test (Student's *t*-test) was carried out. The results are also shown in Table 4. As calculated *P*-values calculated were >0.05 (5%) in all cases, the null hypothesis appears to be valid, i.e., recoveries are close to 100%.

Inter-day precision (expressed as relative standard deviation, RSD) was lower than 14%. Therefore, all compounds were within the acceptable limits for bioanalytical method validation, which are considered $\leq 15\%$ of the actual value, except at the LOQ, which it should not deviate by more than 20%. These data (shown in Table 4) demonstrated that the proposed method is highly reproducible.

Precision and trueness data indicate that the methodology to determine the target compounds in human serum samples is highly accurate, and that the presence of co-extracted matrix components, which typically suppress the analyte signal in mass spectrometry, did not affect the performance of the method.

Table 4
Recovery assay, precision and trueness of the proposed analytical method for the target compounds in fetal bovine serum.

	Spiked (ng mL^{-1})	Found[a] (%, RSD)	Recovery (%)	t_{calc}
4-OH-BP	2.0	2.0 ± 0.1(9.6)	102	1.03
	20	19 ± 1(9.5)	97	1.45
	40	40 ± 1(3.3)	100	0.14
BP-1	2.0	2.1 ± 0.1(13.7)	105	1.66
	20	19 ± 1(7.6)	97	1.63
	40	41 ± 1(4.8)	101	1.15
BP-2	2.0	2.1 ± 0.1(10.2)	103	1.28
	20	20 ± 1(5.6)	98	1.55
	40	39 ± 2(9.0)	98	0.96
BP-3	2.0	2.1 ± 0.1(12.1)	106	1.98
	20	20 ± 1(8.4)	99	0.69
	40	41 ± 2(8.1)	102	0.81
BP-6	2.0	2.0 ± 0.1(4.9)	97	1.16
	20	20 ± 1(1.9)	101	1.23
	40	41 ± 1(4.7)	102	1.66
BP-8	2.0	2.1 ± 0.1(8.9)	104	1.78
	20	20 ± 1(4.3)	99	0.13
	40	41 ± 2(6.8)	103	1.79

[a] Mean of 18 determinations (ng mL^{-1}) ± confidence interval; RSD, relative standard deviation.

Table 5
Results obtained after application of the proposed method to the analysis of human serum samples.

Sample	Form	Concentration (ng mL^{-1})[a]					
		4-OH-BP	BP-1	BP-2	BP-3	BP-6	BP-8
M01	Free	ND	ND	ND	D	ND	ND
	Total	ND	D	ND	0.9	ND	ND
M02	Free	ND	ND	ND	D	ND	ND
	Total	ND	D	ND	0.9	ND	ND
M03	Free	ND	D	ND	ND	ND	ND
	Total	ND	D	ND	1.2	ND	ND
M04	Free	ND	D	ND	D	ND	ND
	Total	ND	D	ND	0.8	ND	ND
M05	Free	ND	D	ND	ND	ND	ND
	Total	ND	D	ND	ND	ND	ND
M06	Free	ND	D	ND	ND	ND	ND
	Total	ND	D	ND	ND	ND	ND
M07	Free	ND	D	ND	ND	ND	ND
	Total	ND	D	ND	ND	ND	ND
M08	Free	ND	ND	ND	ND	ND	ND
	Total	ND	ND	ND	ND	ND	ND
M09	Free	ND	ND	ND	ND	ND	ND
	Total	ND	ND	ND	1.1	ND	ND
M10	Free	ND	D	ND	ND	ND	ND
	Total	ND	D	ND	ND	ND	ND
M11	Free	ND	D	ND	ND	ND	ND
	Total	ND	0.7	ND	D	ND	ND
M12	Free	ND	D	ND	ND	ND	ND
	Total	ND	D	ND	D	ND	ND
M13	Free	ND	D	ND	ND	ND	ND
	Total	ND	D	ND	D	ND	ND
M14	Free	ND	D	ND	ND	ND	ND
	Total	ND	D	ND	D	ND	ND
M15	Free	ND	D	ND	ND	ND	ND
	Total	ND	D	ND	1.0	ND	ND
M16	Free	ND	D	ND	ND	ND	ND
	Total	ND	D	ND	1.1	ND	ND
M17	Free	ND	D	ND	ND	ND	ND
	Total	ND	D	ND	ND	ND	ND
M18	Free	ND	D	ND	0.7	ND	ND
	Total	ND	D	ND	1.2	ND	ND
M19	Free	ND	D	ND	ND	ND	ND
	Total	ND	D	ND	D	ND	ND
M20	Free	ND	D	ND	ND	ND	ND
	Total	ND	D	ND	D	ND	ND

ND: not detected (< LOD); D: detected (> LOD and < LOQ).

[a] Mean of 3 determinations.

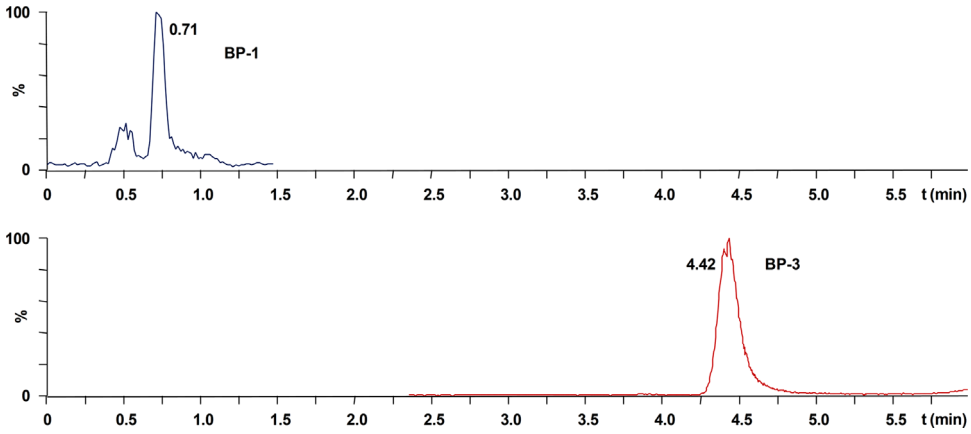

Fig. 3. SRM mode chromatogram of a real serum human sample.

3.4. Method application

The proposed method was applied to the determination of free and total benzophenone-UV filters concentrations in 20 human serum samples from unknown men and women living in the city of Linares (Jaén, Spain). All samples were analyzed in triplicate. The results obtained as mean of three determinations are summarized in Table 5. Fig. 3 shows the SRM chromatogram obtained for a real human serum.

As it is shown in Table 5, only BP-1 and BP-3 were detected and quantified in that group of samples. BP-1 was detected in almost all samples ($n=18/20$), but it was only quantified in one of them. BP-3 was detected in 70% of analyzed samples ($n=14/20$), and quantified in 40% ($n=8/20$).

There were differences between free and total forms of BP-3. Free form was hardly detected ($n=3/20$), but total form could be detected in the majority of the samples and quantified in 8 of them. These data show that conjugated BP-3 is the most predominant form in human serum. On the other hand, there were not significant differences between free and total forms of BP-1. Furthermore, it seems to be a relationship about the presence of BP-3 and BP-1 in the analyzed samples: in all the samples which had a detected BP-3 concentration there was also a detected concentration of BP-1. These facts suggest a possible transformation of BP-3 in BP-1 (see Section 1), so that the content of BP-1 may be due to human metabolism and not to a direct exposure.

The results obtained are in agreement with previously published data on the presence and determination of BP-UV in biological samples. Zhang et al. [31] detected BP-3 in the 83% of the analyzed blood samples (19/23), being the concentration range higher than the one observed in the present study. However, Ye et al. [33] did not find any detectable concentration of BP-3 in a group of 15 serum samples. Due to a lack of demographic and consume pattern information, the reasons of these differences could not be easily explained. Nevertheless, the prevalence of BP-1, as metabolite of BP-3, observed in the present paper is in agreement with the results reported by Tarazona et al. [34].

On the other hand, it has been observed that urinary concentrations of BP-UV filters are significantly higher than the concentrations found in serum samples, probably due to its rapid metabolization [7].

4. Conclusions

The identification and quantification of free and total concentration of six BP-UV filters in human serum samples was successfully performed using a DLLME–UPLC–MS/MS method. The isolation of analytes from serum samples was accurately optimized and the procedure was validated. The proposed method has been used for determination of these compounds (free and total content) in 20 samples collected from men and women living in the city of Linares (Spain). This is a fast and simple analysis method that can be used in further studies for the determination of human exposure to BP-UV filters.

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