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Determination of benzophenone-3 and its main metabolites in human serum by dispersive liquid–liquid microextraction followed by liquid chromatography tandem mass spectrometry

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ABSTRACT

A new analytical method for the determination of benzophenone-3 (2-hydroxy-4-methoxybenzophenone), and its main metabolites (2,4-dihydroxybenzophenone and 2,2'-dihydroxy-4-methoxybenzophenone) in human serum is presented. The method is based on dispersive liquid–liquid microextraction (DLLME) as preconcentration and clean-up technique, followed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Acidic hydrolysis and protein precipitation with HCl 6 M (1:1) (100 °C, 1 h) were carried out before extraction. The variables involved in the DLLME process were studied. Under the optimized conditions, 70 μL of acetone (disperser solvent) and 30 μL of chloroform (extraction solvent) were mixed and rapidly injected into 800 μL of hydrolyzed serum sample. Sample pH or ionic strength adjustment were not necessary. The method was validated by analyzing spiked human serum samples. No satisfactory recoveries were obtained when aqueous standards or standards prepared in synthetic serum were used, but excellent recoveries were achieved by using matrix-matched calibration standards. Moreover, limits of detection in the low μg L^{-1} level and good repeatability were obtained. In order to show the applicability of the proposed method in the study of percutaneous absorption processes, it was applied to the analysis of serum samples from two volunteers after topical application of a sunscreen cosmetic product containing 2-hydroxy-4-methoxybenzophenone.

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1. Introduction

2-hydroxy-4-methoxybenzophenone (HMB), also known as benzophenone-3, has been used for many years as an UV filter in sunscreen cosmetic products, mainly due to its large molar absorptivity in both UVA and UVB ranges. Its use is allowed in the three main regulatory legislations on cosmetic products over the world [1], i.e. the European Union Cosmetics Directive (currently recast as the new Regulation on Cosmetic Products [2]), the United States Food and Drug Administration and the Japanese Pharmaceutical Affairs Law. However, the concentration of the UV filters in the final product is restricted by these legislations. Specifically, the use of HMB in the final product is allowed up to 10%, 6% and 5% (w/w), respectively [1].

UV filters are considered safe for topical application on human skin when employed in the established conditions. However, there are some studies that reveal that after dermal application of sunscreen cosmetic products, UV filters are absorbed through the skin, metabolized in the human body and finally excreted [3]. The percutaneous absorption of these compounds may result in different adverse health effects, such as photoallergy, endocrine disruption or carcinogenicity [4–8]. Specifically, HMB can be considered a potential allergen and contact photoallergen [4,9,10]. Moreover, different *in vitro* studies using human cell lines show that daily exposure to formulations containing HMB might have antiandrogenic and estrogenic activity in humans [5,7,11].

Regarding HMB metabolism, it was first proposed by Okereke et al. after oral [12] and dermal [13] administration to rats. Analysis of biological fluids and tissues revealed the formation of two phase I metabolites mainly, 2,4-dihydroxybenzophenone (DHB) and 2,2'-dihydroxy-4-methoxybenzophenone (DHMB), and a third one in a much lesser extent named 2,3,4-trihydroxybenzophenone (THB). These authors also found their corresponding phase II glucuronide conjugates.

It should be mentioned that metabolites remain in the human body longer than their parent compound, thus its adverse effects may be more important [14]. In fact, it has been shown that DHB has higher antiandrogenic activity than HMB [15] and both have similar estrogenic behavior than other well-established endocrine disruptors, such as bisphenol A, methoxychlor, endosulfan or dibutylphthalate. Moreover, they also shows synergistic effects when several UV filters are

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applied concurrently [11]. Furthermore, it has been shown that DHMB also displays estrogenic activity [16].

Studies about the pharmacokinetics of HMB showed its presence in blood stream, breast milk, semen and different tissues, being urine the major route of excretion. The literature concerning the analytical methods developed to determine this compound and its metabolites in biological fluids and tissues has been recently compiled in a review article written by some of the authors of the present work [3], to which more interested readers are referred to.

Regarding the determination of HMB, individually or together with its metabolites, in serum samples, different methods have been published, mostly based in liquid chromatography (LC), either with UV/Vis [12,13,17–25] or mass spectrometry (MS) detection [26]. Gas chromatography (GC) has been less employed due to the low volatility of these compounds. Derivatization by sylilation previous to the injection was required in this case [14].

It should be emphasized that, from a pharmacokinetic standpoint, in order to carry out a reliable determination of the target compounds, it is mandatory to hydrolyze the phase II glucuronide conjugates although only the free form of these compounds presents estrogenic activity. If not, only the free content would be determined and the conjugated fraction would be obviated. In any case, the difference between the total content (with hydrolysis step) and the free content (without hydrolysis step) results in the conjugated content. This step can be performed with HCl and heating (acidic hydrolysis) [12–14,19,20] or with β-glucuronidase/sulfatase (enzymatic hydrolysis) [26]. The acidic hydrolysis breaks the bound of the target compounds with glucuronic acid and also causes the protein denaturation, with its subsequent precipitation. In enzymatic hydrolysis, organic solvents, mainly pure acetonitrile or mixed with ethanol, need to be added to serum with the purpose of protein and enzyme precipitation [26].

Taking into account that serum is a complex matrix where the target compounds are at trace levels, the hydrolyzed serum without proteins is usually subjected to an extraction step to eliminate potentially interfering compounds and to preconcentrate the target compounds. In this sense, liquid–liquid extraction (LLE) [12–14,18] and solid phase extraction (SPE) [24–26] have been employed in some cases. However, no evidences of the use of the highly-potential microextraction techniques have been found. Among them, it should be emphasized the so-called dispersive liquid–liquid microextraction (DLLME), developed by Assadi and coworkers in 2006 [27]. The fundament of the DLLME has been explained elsewhere, as well as the advantages over the traditional extraction techniques and other microextraction techniques [27,28].

Finally, it is important to note that the *in vivo* experiments performed to determine phase I metabolites of HMB in plasma or serum has been carried out only by means of experimental animals (rats or piglets) [12–14,18,21,22]. *In vivo* studies in humans have only been carried out for the determination of the parent compound [20,23,26,29]. Taking into account that the European legislation forbids the use of experimental animals to evaluate the effectiveness or safety of cosmetic products, it is necessary to advance in the development of new analytical methods to study the processes of absorption and/or excretion of the UV filters without the use of experimental animals.

In order to contribute with the study of the percutaneous absorption of HMB, the aim of this work is to develop an analytical method to determine the total (i.e., free plus conjugated) content of HMB and its main metabolites (DHB and DHMB) in human serum, based on DLLME as microextraction technique before LC-MS/MS determination. The minor metabolite, i.e., THB, was finally excluded from this study because it was not efficiently nor repeatably extracted from serum by DLLME, probably due to its higher polarity. In any case, it has been shown that it is formed in negligible amounts in animal plasma compared to the other two main metabolites [13,14,21,22].

2. Experimental

2.1. Reagents and samples

2-Hydroxy-4-methoxybenzophenone (HMB) 98%, 2,4-dihydroxybenzophenone (DHB) 99% and 2,2'-dihydroxy-4-methoxybenzophenone (DHMB) 98% from Sigma-Aldrich (Steinheim, Germany) were used as standards. dihydroxy-4,4'-dimethoxybenzophenone (DHDMB) 98% also from Sigma-Aldrich was used as surrogate.

LC-grade absolute ethanol from Scharlab (Barcelona, Spain) was used as solvent to prepare the standard stock and working solutions. De-ionized water, obtained by means of a NANOpure II water purification system from Barnstead (Boston, USA), LC-grade methanol (MeOH) also from Scharlab and formic acid from Fluka (Steinheim, Germany) were used for the mobile phase.

Analytical reagent-grade chloroform and analytical reagent-grade acetone, both from Scharlab, were used as extraction and disperser solvent, respectively.

Analytical reagent-grade sodium chloride (NaCl) 99.5% from Scharlab was used in the study of the ionic strength.

Formic acid from Fluka and acetic acid, trichloroacetic acid, sulfuric acid, hydrochloric acid and acetonitrile, all from Scharlab, were tested to protein precipitation.

Sodium chloride, sodium di-hydrogen phosphate monohydrate from Panreac (Barcelona, Spain) and di-sodium hydrogen phosphate dodecahydrate from Scharlab were used to prepare synthetic serum.

Typical cosmetic-grade ingredients from Guinama S.L. (Valencia, Spain) such as emollients, hydrating agents, preservatives, etc. were used to prepare a laboratory-made cream, according to an adapted procedure [30].

Nitrogen (99.9%), used as nebulizing and desolvation gas in the ESI source, was provided by a high-purity generator (CLAN Tecnológica, Sevilla, Spain). Argon (99.9992%) (Carburos Metálicos, Paterna, Spain) was used as collision gas in the MS system.

Blank serum samples used for the method development and validation were obtained from different healthy volunteers who were known not to use cosmetic products containing HMB. Moreover, serum samples from other healthy volunteers who topically applied a laboratory-made sunscreen cream containing 5% of HMB were employed for method application. Each volunteer gave written informed consent to participate in this study, which was conformed to the ethical guidelines of the Declaration of Helsinki, and was approved by the Ethical Committee of the University of Valencia (Spain).

2.2. Preparation of synthetic serum

Synthetic serum was prepared by diluting NaCl (2.4 g), $NaH_2PO_4 \cdot H_2O$ (1.2 g) and Na_2HPO_4 (4.3 g) in 1 L of deionized water.

2.3. Serum samples obtention

Blood extractions were carried out into non heparinized tubes. After extraction, the tubes were centrifuged at 5000 rpm during 15 min to obtain serum. All the serum samples were stored at -20 °C in the freezer until the analysis.

2.4. Proposed method

2.4.1. Preparation of standard solutions and external calibration

Multi-component (HMB, DHB and DHMB) and surrogate (DHDMB) stock solutions were prepared separately at 200 mg L^{-1} in ethanol. From these solutions, working solutions were prepared daily at 10 mg L^{-1} in the same solvent. Both solutions were kept at 4 °C in the refrigerator.

Matrix-matched calibration was prepared by transferring six aliquots of 600 μL of a blank serum to 1.5 mL Eppendorf tubes, which were respectively spiked to achieve concentrations of the target analytes from 0 to 200 μg L^{-1} (note: in order to have a representative blank serum, it is recommended to pool different blank serum samples, thus minimizing the small differences that could exist between different serums). The surrogate (DHDMB) was added at 200 μg L^{-1} in all the calibration solutions.

2.4.2. Acidic hydrolysis and protein precipitation

Both matrix-matched calibration and sample solutions (600 μL) were treated with 600 μL of HCl 6 M to carry out the acidic hydrolysis and protein precipitation. After vortex shaking, the hydrolysis was performed keeping the solutions at 100 °C during 1 h in the oven (note: if only the free content would like to be determined, no heating should be performed). After that, centrifugation during 10 min at 15,000 rpm, to ensure the complete separation of the proteins, was performed. Then, the supernatant was subjected to the DLLME step.

2.4.3. DLLME procedure

After centrifugation, 800 μL of the supernatant obtained were placed into a 1.5 mL Eppendorf tube and subjected to the DLLME process. Thus, a mixture of 70 μL of acetone (disperser solvent) and 30 μL of chloroform (extraction solvent) was rapidly injected into the standard/sample solution employing a 250 μL Hamilton (Bonaduz, Switzerland) 825 RNW syringe. The formed cloudy solution was vigorously shaken with vortex mixer during 3 s. Finally, it was centrifuged during 5 min at 6000 rpm. After phase separation, all the sedimented phase (15–20 μL) was collected and transferred into a 1.5 mL LC injection vial. The extract was dried under a gentle air stream, and then redissolved in 60 μL of MeOH: 0.1% formic acid (50:50) mixture. The vial was closed and vigorously shaken.

2.4.4. LC-MS/MS analysis

An Acquity UPLC H Class with TQ Detector from Waters (Milford MA, USA) was used for the analysis.

The column used was a Waters Acquity UPLC BEHC18 (2.1 × 50 mm, 1.7 μm). Mobile phase consisted of solvent A (water with 0.1% of formic acid) and solvent B (MeOH). The pumps supplied the following gradient at 0.35 mL min^{-1} and 30 °C: 0–0.15 min, 40% solvent B; 0.15–3 min linear gradient to 95% solvent B, held for 1.7 min; 4.7–4.8 return to 40% solvent B and held for 1.2 min. The injection volume was 5 μL.

The MS detector operated in positive electrospray ionization mode (ESI+) by multiple reaction monitoring (MRM). The ESI probe and ion source in positive ion mode were operated at 3.5 kV of capillary voltage. Source temperature was set at 120 °C, desolvation temperature was 300 °C, nebulizer gas flow rate was 25 L h^{-1} and desolvation gas flow rate was 600 L h^{-1}.

The MS/MS spectra were produced by collision-induced dissociation (CID) of the selected precursor ions. The m/z precursor→product ion transitions for quantification were 229.4→151.0 for HMB, 215.3→136.8 for DHB, 245.5→121.1 for DHMB and 275.4→151.2 for DHDMB; whereas 229.4→104.9 for HMB, 215.3→104.8 for DHB, 245.5→151.1 for DHMB and 275.4→185.1 for DHDMB were used as qualifier transitions.

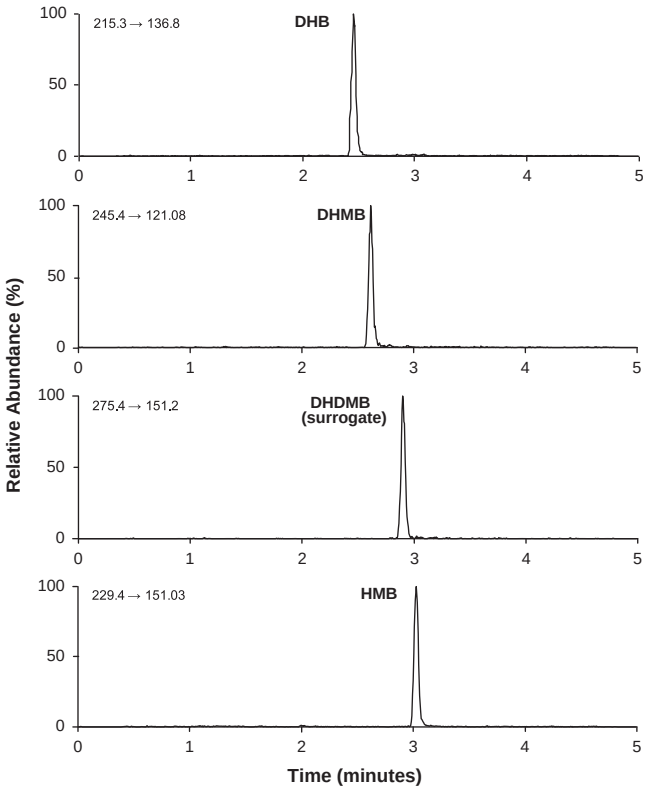


Fig. 1. A typical chromatogram obtained applying the proposed DLLME-LC-MS/MS method to a blank serum spiked with 200 μg L^{-1} of the target analytes and the surrogate (DHDMB) (see text for experimental details). Transitions correspond to: m/z precursor ion→m/z product ion.

Fig. 1 shows a chromatogram of a blank serum standard solution containing the target analytes (200 μg L^{-1}) and the surrogate (200 μg L^{-1}) subjected to the described DLLME-LC-MS/MS procedure.

3. Results and discussion

3.1. Study of the acidic hydrolysis and protein precipitation

Proteins precipitation before sample injection is necessary to reduce matrix interferences and to avoid precipitation in the chromatographic column, caused by the presence of organic solvents in the mobile phase, clogging the chromatographic column.

Formic acid, acetic acid, trichloroacetic acid, sulfuric acid, hydrochloric acid and cold acetonitrile were tested for this purpose. When acetonitrile was used, no cloudy solution was observed when the mixture of disperser and extraction solvents was injected into the solution to carry out the DLLME process. Regarding the acids tested, in most of the cases a small amount of proteins still precipitated when the DLLME process was carried out, as not all the proteins had been previously removed. Only the use of HCl 6M (1:1, acid:sample) allowed the complete proteins precipitation.

Moreover, the addition of HCl enables the determination of the total content of the target compounds (free and bounded form) since acidic hydrolysis occurs if solution is kept at 100 °C during 1 h.

3.2. Study of the experimental variables involved in the DLLME procedure

The different variables that may affect the DLLME process, such as the type and volume of both extraction and disperser solvents, the ionic strength and the pH of the sample, as well as the extraction time, were studied.

In this sense, a blank serum solution spiked with 100 μg L^{-1} of the target analytes was used to carry out the DLLME optimization. The influence of the variables involved in the process was evaluated in terms of the analytical signal, corresponding to the peak area obtained for each of the target analytes.

3.2.1. Considerations on the extraction solvent and disperser solvent

The selection of the extraction solvent and disperser solvent is a key issue in the DLLME process. On one hand, the extraction solvent should extract the target analytes efficiently and have low solubility in the aqueous phase. Moreover, as only a few microliters of extraction solvent are employed, a solvent with a higher density than water is recommended in order to remain in the bottom of the extraction tube and ease its collection. On the other hand, the disperser solvent should be miscible in both, aqueous sample and organic extraction solvent, and should also form the so-called cloudy solution. Moreover, after centrifugation, phase separation has to be achieved.

The optimum disperser–extraction solvent mixture for the extraction of the target analytes in aqueous samples was previously studied by some of the authors of the present work [28]. A binary study considering acetone, ethanol and acetonitrile as disperser solvents, and chloroform, dichloromethane and carbon tetrachloride as extraction ones, was carried out. The best results were obtained employing the acetone-chloroform combination, thus this solvents were employed in the present work.

3.2.2. Effect of the extraction solvent volume

It is assumed that increasing the extraction solvent volume will increase the amount of the analytes extracted. However, the dilution effect should be taken into account. Thus, a careful study was carried out in order to reach the best results.

In this sense, 200 μL of an acetone–chloroform mixture containing different volumes of chloroform, ranging from 5 to 30 μL, were tested. As the extraction solvent volume increased, the volume of the sedimented phase also increased, ranging from 5 to 15 μL approximately. When 5 μL of chloroform were employed, the cloudy solution was not observed. Moreover, no phase separation occurred after centrifugation. In the case of 10 μL of chloroform, a pale cloudy solution was formed but phase separation did not occur. In all the other cases, an adequate phase separation was obtained. As can be seen in Fig. 2, the use of 15 μL of chloroform provides the worst results. No significant differences were observed when 20, 25 and 30 μL of this extraction solvent were employed. With the purpose to ease the sedimented phase collection, a volume of 30 μL of chloroform was selected.

3.2.3. Effect of the disperser solvent volume

In order to study the effect of the disperser solvent volume, different volumes of acetone as disperser solvent, ranging from 60 to 220 μL, were tested. When 60 μL of acetone was used, no cloudy solution was formed. As can be seen in Fig. 3, small differences were observed when the disperser solvent volume ranged between 70 and 220 μL. A volume of 70 μL of acetone was finally chosen, thus reducing the amounts of organic solvent employed.

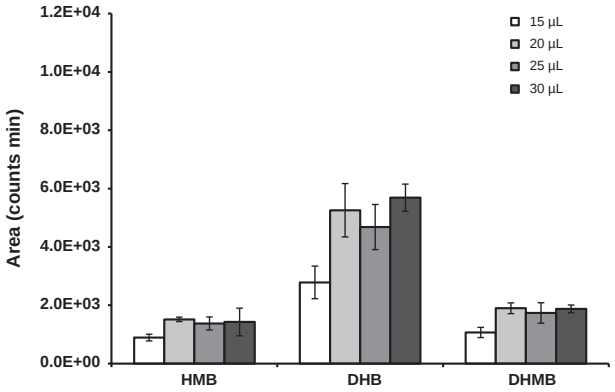


Fig. 2. Effect of the extraction solvent volume on the DLLME process (extraction conditions: 800 μL of sample solution, 200 μL of acetone-chloroform mixtures containing different volumes of chloroform as extraction solvent).

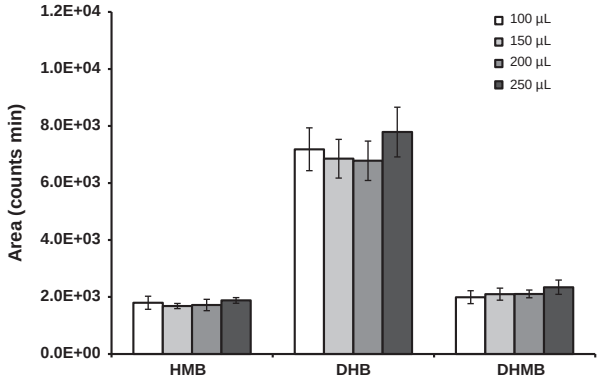


Fig. 3. Effect of the disperser solvent volume on the DLLME process (extraction conditions: 800 μL of sample solution, different volumes of acetone as disperser solvent volumes mixed with 30 μL of chloroform as extraction solvent).

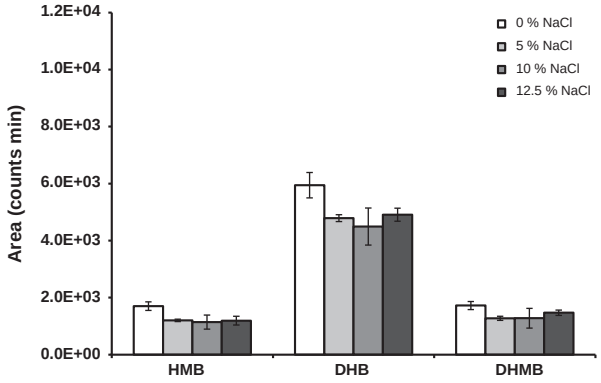


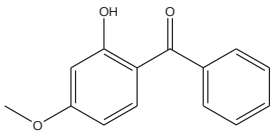
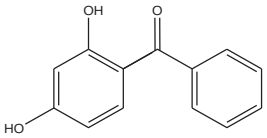
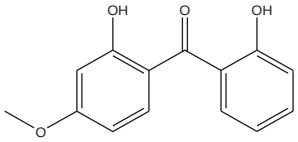
Fig. 4. Effect of the ionic strength of the donor phase on the DLLME process (extraction conditions: 800 μL of sample at different ionic strength values, 70 μL of acetone as disperser solvent mixed with 30 μL of chloroform as extraction solvent).

3.2.4. Effect of the ionic strength

In general terms, the addition of salt to the aqueous sample reduces the solubility of the organic compounds in water and forces them to pass to the extraction solvent (*salting-out* effect). Hence, the effect of the sample ionic strength in the analytical signal was studied.

Taking into account the serum salt content (0.9%), NaCl was added to the donor phase at the adequate amounts to reach final concentration values up to 12.5% (m/v). 15% of NaCl was tested but the salt was not completely dissolved in the hydrolyzed serum at this concentration. Contrary to the expected, the best results were

Table 1
Structure and some relevant data of the target compounds.

Analyte	Chemical structure	Molecular formula	CAS number	pKa[a]
2-hydroxy-4-methoxybenzophenone (HMB)		$C_{14}H_{12}O_3$	131-57-7	7.56 ± 0.35
2,4-dihydroxybenzophenone (DHB)		$C_{13}H_{10}O_3$	131-56-6	7.53 ± 0.35
2,2'-dihydroxy-4-methoxybenzophenone (DHMB)		$C_{14}H_{12}O_4$	131-53-3	6.99 ± 0.35

[a] Calculated using Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris (©1994–2010 CD/Labs).

obtained without salt addition (Fig. 4). This is probably due to the fact that the salt addition decreases the chloroform solubility in the aqueous phase, which causes an increase in the sedimented phase volume. Thus, the concentration of the target analytes in this phase decreases due to the dilution effect. Moreover, vigorously vortex agitation was necessary to dissolve NaCl in the serum sample, causing bubbles which were then difficult to remove and can decrease the extraction efficiency. Therefore, no salt adjust was necessary in this process.

3.2.5. *Effect of the pH*

The pH of the donor phase is a variable of interest in case of potentially ionizable compounds, since the neutral form of a molecule is expected to be extracted in the organic extraction solvent more efficiently than the ionic form. The addition of an acidic or basic compound in a small sample volume (800 μL in this case) will involve a high sample dilution and thus an increase of the limits of detection (LODs) of the method. As HCl 6 M is added to the blood samples to enable protein precipitation (see Section 2.4.2), the sample subjected to the DLLME process presents an acidic pH, so the analytes are in the neutral form, the proper to be extracted (see pK_a values in Table 1). Thus, the sample pH was not adjusted before de DLLME process.

3.2.6. *Effect of the extraction time*

The extraction time is one of the most important variables involved in the extraction procedures. In DLLME, the extraction time is defined as the interval time between the injection of the binary mixture of disperser and extraction solvents and just before starting to centrifuge [27]. In this modality of extraction, the surface area between the extraction solvent and the aqueous donor phase is extremely large, thus the transfer of the target analytes from the aqueous donor phase to the extraction solvent phase is fast, achieving the equilibrium state quickly. For this reason, it is expected that the extraction time does not influence the extraction efficacy. A study from 0 to 15 min was carried out to confirm it. Results (not shown) revealed that the extraction time had no effect on the response for any of the target analytes.

3.3. *Study of matrix effects*

In order to evaluate matrix effects, a comparison study between deionized water, synthetic serum and human serum was performed. For this, a set of calibration solutions was made in each case and then the solutions were subjected to the DLLME method and finally measured. The obtained slopes were compared with a Student's *t*-test [31]. As can be seen in Table 2, significant differences were observed for a 5% significance level between the slopes in water (or synthetic serum) and real serum. Thus, external calibration using deionized water or synthetic serum should not be used for quantification purposes since a severe and negative matrix effect is observed. Taking into account that an extraction (i.e., DLLME) is performed, this negative matrix effect could be attributed to the differences in the extraction efficiency when the target analytes are extracted from water, synthetic serum or real serum, instead of the well-known ion suppression effect that might be encountered in LC-MS/MS analysis. Thus, in order to correct this matrix effect, standard addition calibration should be conducted.

Table 2
Matrix effect study on the determination of the target compounds.

Analyte	Matrix type	Slope ± deviation (counts min μg^{-1} L) [a]	Student's *t*-test[b]	
			t_{exp}	Equality
HMB	Deionized water	110 ± 10	9.73	No
	Synthetic serum	22.0 ± 0.8	23.65	No
	serum	25 ± 2	–	–
DHB	Deionized water	620 ± 30	19.72	No
	Synthetic serum	300 ± 20	11.93	No
	Serum	99 ± 3	–	–
DHMB	Deionized water	300 ± 10	22.77	No
	Synthetic serum	63 ± 4	9.84	No
	Serum	25 ± 2	–	–

[a] Working range: 0–250 μg L^{-1}. Number of calibration points: 6.
[b] t_{exp}: Student´s *t*-test for slopes comparison with real serum ($t_{crit(0.05,4)}$=2.77).

However, standard addition calibration requires the preparation of a different set of calibration solutions for each sample that widely increases the preparation time when a high number of samples have to be analyzed. With the aim to avoid this calibration methodology, matrix-matched calibration, i.e, spiked blank serum samples as calibration standards, was assayed. In this sense, four serum samples coming from four volunteers who were known not to use cosmetics containing HMB were used to construct different calibration sets. For each target analyte, the obtained slopes in

each one of the calibration sets were compared. As can be seen in Table 3, the slopes obtained with the four blank serums are statistically comparable for a 5% significance level by applying an ANOVA test [31]. With the aim to obtain a representative serum blank, the four serum blank samples were pooled, and the resultant was used to construct a calibration set. The individual calibration sets were compared with the pool. Table 3 shows the Student's *t*-test values obtained by comparison of the slope of each individual serum and the one obtained employing a pool of all serums. As expected, results indicate that they were statistically comparable at a 5% significance level. Therefore, it can be concluded that matrix-matched calibration could be used for the determination of HMB and metabolites in human serum. Once at this point, it should be said that the more individual serum samples are taken to form the pool, the more representative it is.

3.4. Use of surrogate

Under optimized conditions, the volume of the sedimented phase ranges between 15 and 20 μL, which causes inadmissible signal variations around 25%. Moreover, the handling of low volumes by the operator could increase this variability even more. In order to reduce this variability, the use of a surrogate was considered, which was included in the serum samples before sample treatment. DHDMB was selected as surrogate for various reasons: (1) it belongs to the same family of the target compounds; (2) it is extractable in chloroform by the DLLME proposed method; and (3) it is not expected to be in the serum samples.

In this sense, A_i/A_s (where A_i is the peak area of the target analyte and A_s that of the surrogate) was used as response function for quantification purposes instead of A_i, decreasing the variability below 10%.

Table 3
Comparison of each individual serum with a pool of all serums.

Analyte	Serum sample	Slope ± deviation (counts min μg^{-1} L) [a]	ANOVA test [b]		Student's *t*-test [c]	
			F_{exp}	Equality	t_{exp}	Equality
HMB	1	25 ± 2	1.94	Yes	0.01	Yes
	2	27 ± 2			0.71	Yes
	3	25 ± 2			0.01	Yes
	4	25 ± 2			0.01	Yes
	Pool	25 ± 2	–	–	–	–
DHB	1	94 ± 3	3.33	Yes	0.26	Yes
	2	101 ± 6			0.54	Yes
	3	103 ± 2			0.96	Yes
	4	101 ± 6			0.54	Yes
	Pool	96 ± 7	–	–	–	–
DHMB	1	24 ± 2	2.01	Yes	0.35	Yes
	2	25 ± 2			0.01	Yes
	3	26 ± 2			0.35	Yes
	4	24 ± 2			0.35	Yes
	Pool	25 ± 2	–	–	–	–

[a] Concentration range: 0–250 μg L^{-1}. Number of calibration points: 6.
[b] ANOVA test for comparison between the four individual serum samples ($F_{crit(0.05,6,16)}$=3.34).
[c] Student´s *t*-test for slopes comparison between each individually serum and the serum pool ($t_{crit(0.05,4)}$=2.77).

3.5. Analytical figures of merit of the proposed DLLME-LC-MS/MS method

Quality parameters of the proposed method were evaluated under final optimized conditions.

The achieved enrichment factors (EF), defined as EF=C_{sed}/C_0, where C_{sed} is the concentration of the target compound in the organic sedimented phase and C_0 is the initial concentration of this compound in the aqueous phase, were 3.1 ± 0.2 for HMB, 7.4 ± 0.6 for DHB and 3.4 ± 0.1 for DHMB. The maximum EF value that could be obtained, corresponding to a total transfer of the target analytes from the aqueous donor phase to the extraction solvent phase, is calculated as V_0/V_{sed}, where V_0 is the sample volume and V_{sed} the sedimented phase volume. In the present work the collected V_{sed} was evaporated and the residue obtained was redissolved in 60 μL of MeOH: 0.1% formic acid (50:50) mixture, so this volume corresponds to V_{sed} in the above relation. Thus, the maximum EF value that could be obtained in the present method corresponds to a value of approximately 13. Although these are relatively low values, they are useful to achieve the determination of the target analytes in real samples (see Section 3.6). Nevertheless, it should not be forgotten that also an efficient clean-up is achieved.

The calibration parameters, obtained employing a blank serum pool (from 0 to 250 μg L^{-1}) from four volunteers, and plotting the ratio of the peak area of each target analyte to the surrogate (DHDMB) (A_i/A_S) versus the analyte concentration, are shown in Table 4. As can be seen, a high level of linearity was obtained in all cases. The linearity studied reached at least 5000 μg L^{-1} in all cases.

The limits of detection (LOD) and quantification (LOQ) of the target analytes in the serum samples are also shown in Table 4 and were found to be in the low μg L^{-1} level, ranging from 7 to 8 μg L^{-1} and from 22 to 28 μg L^{-1}, respectively. These values are lower that those reported by Kasichayanula et al. by using LC-UV [21], in the low mg L^{-1} range; whereas are of the same order of those reported by Jeon et al. by using LLE-GC-MS [14], showing the capacity of the present method to achieve the determination of low levels of the target analytes in this complex matrix.

The repeatability, expressed as relative standard deviation (RSD), was studied at two concentration levels. The proposed method was applied to five replicates of serum blanks spiked at 50 μg L^{-1} (low concentration level) and at 200 μg L^{-1} (high concentration level). The obtained repeatability values ranged

Table 4
Main parameters of the proposed DLLME-LC-MS/MS method for the determination of HMB and its main metabolites in human serum.

Analyte	Slope ± deviation [a] (μg^{-1} L)/10^4	Intercept ± deviation [a]	Regression coefficient (R^2) [a]	LOD [b] (μg L^{-1})	LOQ [b] (μg L^{-1})	Repeteability [c] RSD (%)	
						Low conc	High conc
HMB	82 ± 1	−0.03 ± 0.02	0.9991	8	28	9	8
DHB	352 ± 6	0.13 ± 0.09	0.9990	8	27	8	6
DHMB	90 ± 1	0.02 ± 0.02	0.9992	7	22	9	4

[a] Concentration range: 0–250 μg L^{-1}. Number of calibration points: 6.
[b] LOD: limit of detection; LOQ: limit of quantification; calculated as 3S_a/*b* and 10S_a/*b* criteria, respectively, where S_a is the intercept standard deviation and *b* is the slope of the calibration curve [32].
[c] Relative standard deviation (RSD); five replicate analysis of spiked blank serum solution (50 μg L^{-1} (low concentration) and 200 μg L^{-1} (high concentration)).

Table 5
Recovery values obtained by applying the proposed DLLME-LC-MS/MS method with matrix-matched calibration to four serum samples.

Sample[a]	Analyte	Added ($\mu g\ L^{-1}$)	Found ($\mu g\ L^{-1}$)	Recovery (%)
A	HMB	178.5	186.0	104
	DHB	156.0	133.0	85
	DHMB	153.0	138.8	91
B	HMB	178.5	166.1	93
	DHB	156.0	140.0	90
	DHMB	153.0	157.3	103
C	HMB	178.5	145.0	81
	DHB	156.0	134.4	86
	DHMB	153.0	137.3	90
D	HMB	178.5	137.0	77
	DHB	156.0	150.7	97
	DHMB	153.0	128.4	84

[a] The initial found amounts are below the LOD.

between 4 and 9%, which are similar values to those reported by other authors at higher concentrations [14,21], showing that a good precision was achieved for all the target analytes at these low levels of concentration.

Recovery studies were conducted by spiking (at 150 $\mu g\ L^{-1}$) four serum samples from four volunteers who were known not to use cosmetics containing HMB. Results (Table 5), as expected, reveal no matrix effects when using matrix-matched calibration.

3.6. Application of the proposed DLLME-LC-MS/MS method to the analysis of real samples

Specific amounts of a laboratory-made sun cream containing 5% of HMB were topically applied to the body of two volunteers after informed consent. 20 and 30 g were applied to Volunteer A (VA, male) and Volunteer B (VB, female) respectively. Each volunteer applied the cream all over the body in the same way as if they were sunbathing on the beach. This dose is included in the usual range of thickness application for sunscreens (0.5–1 mg of cream per cm^2 of skin), which is usually below the recommended dose for a maximum sun protection (2 mg/cm^2) [33]. Blood samples were collected both before and after the application at different time intervals for a period of 24 h. Then, they were centrifuged, and serum samples were kept at −20 °C in the freezer until analyzed according to the DLLME-LC-MS/MS proposed method.

As can be seen in Fig. 5, both volunteers present detectable amounts of HMB in serum before cream application (time = 0). This is a reasonable fact since there are many commercially available cosmetic products that contain HMB in its formulation. Regarding the HMB metabolism, both volunteers present a similar profile. Thus, the amounts of HMB significantly increases during the first hours to reach a maximum concentration level ranging between 6 (VA, 200 $\mu g\ L^{-1}$) and 9 h (VB, 304 $\mu g\ L^{-1}$) after the cream application. From this point, the HMB signal slowly decreases. After 24 h of the cream application, high amounts of HMB are still present in the serum of the volunteers (84 $\mu g\ L^{-1}$ for VA and 206 $\mu g\ L^{-1}$ for VB). Regarding metabolites, DHMB is formed at a very small extent, while DHB is detectable from the first hour after the cream application. Concentrations of this metabolite increase among all the study, being this increase slightly more pronounced in the first six hours and reaching concentrations of 34 (VA) and 102 $\mu g\ L^{-1}$(VB) after 24 h of application.

It should be commented that the analysis of a higher number of samples from different people and during a longer time should be carried out using the proposed method to get a more reliable idea of the metabolism of HMB and to carry out toxicokinetics studies. Nevertheless, this is not the aim of this study, which is to develop an analytical method and to show its applicability to carry out reliable toxicokinetic studies.

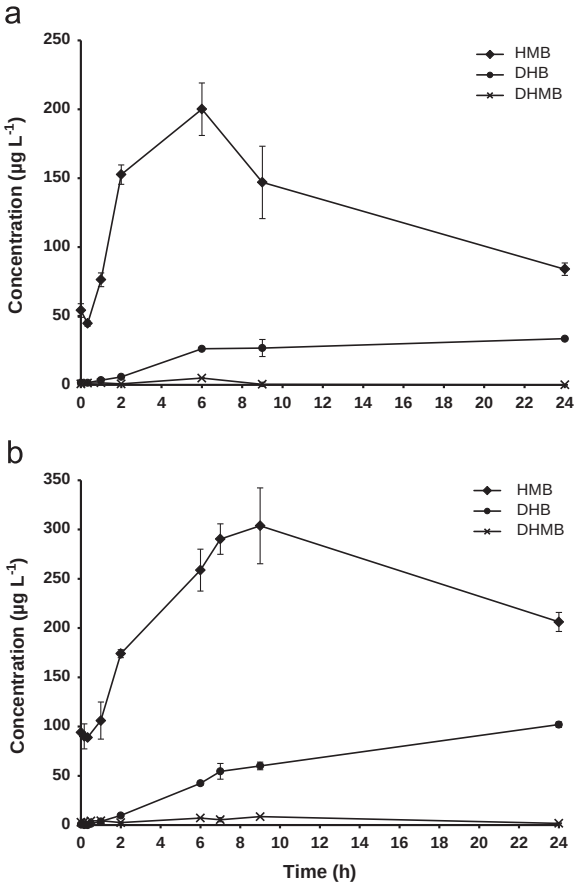


Fig. 5. Metabolic profile of HMB and its main metabolites in the serum of two volunteers ((volunteer A, female (a) and volunteer B, male (b)) after the topical application of a laboratory-made sun cream containing 5% of HMB.

4. Conclusions

A new analytical method to determine HMB and its two main metabolites in human serum has been presented. The method is based on the use of LC-MS/MS with a previous protein precipitation and DLLME. The use of DLLME is needed to clean-up and preconcentrate, since serum samples have a complex matrix which contain many compounds that can interfere and the target compounds are in low levels of concentration in this biological fluid.

The analysis of serum samples employing aqueous external calibration with deionized water or synthetic serum presented severe matrix effects. However, the use of matrix-matched calibration provided excellent results.

When a sunscreen cream containing 5% of HMB was applied to two volunteers, similar metabolism profiles were observed for both of them. After 24 h of the cream application, the HMB concentrations in serum were still appreciable. The main metabolite formed in serum was DHB, which concentrations in serum continue to rise after 24 h of cream application. Regarding DHMB, after cream application, the concentration of this compound increases until it reaches a maximum concentration, after which the analytical signal falls slowly. Thus, after a single application of

a sun cream the complete metabolism or excretion of HMB requires more than one day.

Finally, as commented above, in order to have a reliable idea about the metabolism of this UV filter in humans it would be necessary, not only to extend the study to a larger number of volunteers and during a longer time, but also to extend the study to other biological fluids which are routes of excretion of these compounds, as urine or semen [34].

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