Chapter 8
Determination of Ethylhexyl Dimethyl PABA and its Metabolites in Semen by Automated Solid-Phase Extraction and Liquid Chromatography Tandem Mass Spectrometry

8.1 Introduction

8.1.1 Aim of the Study

Due to the hydrophobicity of EDP, this UV filter can undergo presumably bioaccumulation in humans and stay in the body longer than other hydrophilic xenobiotics, thus being metabolized and/or excreted. This hypothesis is evident from the results obtained in the *in vivo* study regarding the determination of the excreted levels in urine from volunteers that had applied a sunscreen cosmetic product containing EDP (see Chap. 7). In this study, the metabolites of EDP were detected in urine samples collected 8 days after a single application of the cosmetic product (León-González et al. 2011).

The development of long-term toxicological studies is particularly important because of the currently tendency of the cosmetic sector about the inclusión of UV filters not only in sunscreen cosmetic products, but also in other daily products, such as face creams, aftershave products, etc.

This study aims to develop and validate an analytical method to determine EDP and its metabolites in human semen using an automated methodology based on the application of a solid-phase extraction (SPE) procedure prior to liquid chromatography tandem mass spectrometry (LC–MS/MS) to evidence bioaccumulation and provide a basis for assessing the toxicity of this widely used UV filter.

Some content of this chapter has been published in *Anal Bioanal Chem* (2011) 401:1003–1011.

Z. León González, *Percutaneous Absorption of UV Filters Contained in Sunscreen Cosmetic Products*, Springer Theses, DOI: 10.1007/978-3-319-01189-9_8,

8.1.2 Background and Current Status of the Issue

According to the literature consulted, there was no precedent for the determination of UV filters in human semen, except benzophenone-3, whose determination was carried out using the analytical method described in Chap. 5 of this PhD Thesis (León et al. 2010). The analytical method was based on a SPE procedure and subsequent LC–MS/MS analysis, and allowed simultaneously the determination of the parent compound and its metabolites at the ng mL^{-1} level.

8.2 Experimental

8.2.1 Reagents and Samples

Ethylhexyl dimethyl PABA (EDP), also called 2-ethylhexyl 4-(*N,N*-dimethylamino) benzoate, 98 % from Aldrich (Milwaukee) and 4-(*N,N*-dimethylamino) benzoic acid (DMP) 98 % and 4-(*N*-methylamino) benzoic acid (MMP) 97 %, both from Sigma-Aldrich (Schnelldorf) were used as analytical standards. Trihexylamine (THA) from Sigma-Aldrich was used as internal standard.

The solvents used were LC grade methanol (MeOH) and LC grade acetonitrile (ACN), both from Scharlab (Barcelona). Deionized water was obtained from a Milli-Q water purification system (R ≥ 18 mΩ cm) from Millipore (Billerica).

Other reagents used were 85 % (m/v) ortho-phosphoric acid (d = 1,710 g mL^{-1}) from Merck (Darmstadt), ammonium acetate from Sigma (St. Louis), citric acid from Panreac (Barcelona) and formic acid (FA) and 32 % (m/v) ammonium hydroxide (d = 0.910 g mL^{-1}) from Scharlab (Barcelona). ß-glucuronidase enzyme from *Helix pomatia* (type H-1) with activity ≥300,000 U g^{-1} and sulfatase activity ≥10,000 U g^{-1} from Sigma-Aldrich was also used.

Semen samples used to develop and validate the analytical method were obtained from healthy male volunteers who had not applied any cosmetic product containing EDP, according to an official protocol of the World Health Organization for the examination and processing of human semen (WHO Laboratory Manual 2010).

8.2.2 Instruments and Material

An automated SPE-LC–MS/MS system consisted of a Prospekt-2® SPE platform from Spark Holland (Emmen) coupled to an 1200 Series LC System® from Agilent Technologies (Palo Alto) that was connected to a 6460 LC/MS® Triple Quad mass spectrometry detector equipped with a Jet Stream® electrospray ionization source, also from Agilent, were used. The automated Prospekt-2® SPE

platform consisted of an automatic cartridge exchanger (ACE) and a high pressure dispenser (HPD) syringe to distribute the SPE solvents. ACE and HPD were connected to a Midas® autosampler from Spark Holland equipped with a 100 μL loop. 0.25 mm internal diameter PEEK (polyether ether ketone) tube from VICI (Houston) was employed for all connections between valves. The automation of the extraction step was performed using the Sparklink V. 2.10 software.

C18-HD (high density phase of octadecyl chains based on silica, particle size 7 μm) HySphere® SPE cartridges (10 mm long and 2 mm internal diameter) from Spark Holland were used.

A C18 Mediterranean Sea® analytical column (3 μm particle size, 150 mm long, 4.6 mm internal diameter) from Teknokroma (Barcelona) was used. Moreover, a high purity generator from CLAN Technology (Sevilla) provided nitrogen gas used in the ionization source. As collision gas, 99.999 % nitrogen from Air Products (Córdoba) was used.

Data acquisition, control of the SPE analysis sequences and development of qualitative and quantitative analyses were carried out with Agilent MassHunter Workstation software.

Finally, a 2001 MicropH® pH meter from Crison (Alella), Eppendorf® tubes (2.5 mL) from Nirco (Granada), a Sorvall Legend Micro 21R® centrifuge from Thermo Scientific (Barcelona), a thermostated water bath from Selecta (Barcelona) and a 450 digital sonicator (20 kHz, 450 W) from Branson Ultrasonics Corporation (Danbury) with adjustable amplitude and duty cycle, equipped with a cylindrical probe of titanium alloy and 12.7 mm in diameter were also used.

8.2.3 Analytical Method to Determine Ethylhexyl Dimethyl PABA and its Metabolites in Semen

8.2.3.1 Preparation of Solutions

2 mL of semen was mixed in an Eppendorf® tube with 0.3 mL of 1 M orthophosphoric acid to denature the proteins present in the biological matrix. Then, the mixture was centrifuged at 10,000 rpm for 10 min at 5 °C. After discarding the pellet, the pH of the supernatant was adjusted to 4.5 or 5 considered with diluted ammonium hydroxide if needed.

Separate analytes standard (EDP, DMP, MMP) and internal standard (THA) solutions in MeOH (200 μg mL^{-1}) were prepared and stored at 4 °C. From these stock solutions, a solution of THA (0.5 μg mL^{-1}) in deionized water and a solution of EDP, DMP and MMP (2 μg mL^{-1}) in the semen supernatant to be analyzed were prepared.

To correct the error caused by the matrix effects (see Sect. 8.3.2), the standard addition calibration was employed. The calibrate standard solutions were prepared by fortifying five aliquots of semen supernatant (270 μL) containing 0, 20, 40, 60

and 80 μL of the analytes solution (0.5 μg mL^{-1}) to which 100, 80, 60, 40 or 20 μL of semen supernatant was added, respectively, to fix the same final volume in all the calibrate solutions. Furthermore, a volume of 50 μL of THA (2 μg mL^{-1}) was added to each calibrate solution.

8.2.3.2 Ultrasonic Assisted Enzymatic Hydrolysis

0.01 g of enzyme was added to a 5 mL of 1 M ammonium acetate (pH 5), solution which was stored at 4 °C.

To determine the total content of the analytes (i.e., both unconjugated and conjugated forms), 100 μL of the ß-glucuronidase solution was added to each calibrate solutions. Next, these solutions were subjected to an enzymatic hydrolysis treatment assisted by ultrasound to deconjugate the possible glucuronide adducts of the phase I metabolites of EDP. Thus, the calibrate solutions were immersed in a thermostated water bath (37 °C) together with the probe tip, which was located at the same distance from each solution (5 cm) and at a fixed distance from the bath background (5 cm). Hydrolysis of conjugated metabolites of EDP was conducted essentially under the conditions previously obtained and described in Sect. 7.2.3.2. Thus, ultrasonic radiation was applied for 50 min at 35 % duty cycle and 50 % amplitude (converted applied power, 400 W).

8.2.3.3 Automated Solid-Phase Extraction

From the final volume of each semen sample (0.5 mL), an aliquot of 250 μL was mixed with 50 μL of a 1 M citric acid/citrate buffer solution, adjusted to pH 3 with ammonium hydroxide and the mixture was homogenized prior to performing the automated SPE procedure.

Selected SPE cartridges (C18 HD) were automatically solvated with 2 mL of MeOH at 5 mL min^{-1}, conditioned with 2 mL of deionized water at 5 mL min^{-1} and equilibrated with 1 mL of deionized water at 0.5 mL min^{-1}. The cartridges were then loaded with 100 μL of semen solution by propelling 2 mL of deionized water at 0.5 mL min^{-1}. Next, the SPE cartridge was washed with 1 mL of an aqueous methanol 5 % (*v*/*v*) solution at 0.5 mL min^{-1}. Finally, the analytes were eluted by flowing the LC mobile phase through the SPE cartridges for 4.5 min. The eluate from each SPE cartridge was chromatographically separated in the analytical column prior to the detection with the mass spectrometer. Then, the cartridges were washed with 2 mL of MeOH at 5 mL min^{-1} and 2 mL of deionized water at 5 mL min^{-1} for further extractions. The sequence of operations of the automated SPE procedure is summarized in Table 8.1.

Table 8.1 Main features of the automated SPE process

Process	Solution	Volume (mL)	Flow (mL min^{-1})	Comment
Placing new cartridge				C18 HD
Autosampler starting				Sample loading (100 μL)
Solvatation step	MeOH	2	5	
Conditioning step 1	Deionized water	2	5	
Conditioning step 2	Deionized water	1	0.5	
Sample injection	Deionized water	2	0.5	Sample injection (100 μL)
Washing step	Deionized water:MeOH (95:5, *v/v*)	1	0.5	
Elution step	LC mobile phase	3.6	0.8	Along 4.5 min
Post-extraction washing 1	MeOH	2	5	
Post-extraction washing 2	Deionized water	2	5	

8.2.3.4 LC–MS/MS Analysis

The chromatographic separation of analytes was carried out using a mobile phase consisting of deionized water and MeOH:ACN (1:1, *v/v*), both containing 0.2 % FA. The elution gradient outlined in Table 8.2 was used at a flow rate of 0.8 mL min^{-1} and at 20 °C. The analytical column was equilibrated with a post-analysis time of 5 min.

The conditions of the mass spectrometer analyzer and spray chamber were: temperature of gas ionization source, 350 °C; drying gas flow, 10 L min^{-1}; nebulizer gas pressure, 35 psi; nebulizer gas temperature, 380 °C; nebulizer gas flow, 10 L min^{-1}; capillary voltage, 4450 V. The dwell time was set to 70 ms.

The analytes were determined by selected reaction monitoring (SRM) in positive electrospray ionization (ESI^+) mode. The selected confirmation and quantification SRM transitions are shown in Table 8.3.

Calibration curves were obtained by representing the peak area ratio of each analyte to the internal standard (THA) versus the added concentration of analyte.

Table 8.2 Elution gradient of the LC–MS analysis to determine EDP and its metabolites in semen

Time (min)	0.2 % FA in deionized water (%)	0.2 % FA in MeOH:ACN (1:1, *v/v*) (%)
0	60	40
1	60	40
3	0	100
15	0	100

Table 8.3 SRM parameters in positive electrospray ionisation mode to determine EDP and its metabolites in semen

Compound	Parameter			
	First quad voltage (V)	Collision energy (eV)	SRM transition to quantify	SRM transition to confirm
MMP	90	15	151.8 → 136.8	151.8 → 119.9
DMP	90	25	165.8 → 149.8	165.8 → 133.9
EDP	100	15	277.8 → 165.8	277.8 → 150.8
THA[a]	120	20	270.1 → 185.8	270.1 → 102.0

[a] Internal standard

Figure 8.1 shows the SRM chromatograms obtained for each analyte in semen samples containing no analyte that were either fortified (40 ng mL^{-1}; blue line) and unfortified (red line) subjected to the analytical procedure described above.

8.3 Results and Discussion

8.3.1 Study of the Experimental Variables

Most of the conditions used in the automated analytical platform were adapted from those described in Chap. 7. Thus, C18 HD cartridges were selected for subsequent experiments, as they provided the best results for these analytes in the case of urine samples analysis.

As the mechanism of extraction was based on the interaction of analytes with the C18 HD sorbent, semen samples were fortified (1 μg mL^{-1}) and acidified to pH 3 with a 1 M citric acid/citrate buffer solution (pH 3) before the automated SPE procedure. The analytes were stable during the time that the samples remained in the autosampler prior to the analysis. No precipitation of the semen samples despite remaining at pH 3 was observed during the complete sequence of analysis. Acceptable variability was obtained [12.6 % for MMP, 5.7 % for DMP and 10.1 % for EDP, expressed as relative standard deviation (RSD)] for at least 4 h for all analytes.

The influence of the loading volume was examined in the range from 0.5 to 5 mL. A volume of 2 mL of deionized water was selected because larger volumes caused the partial elution of MMP and lower volumes did not allow the loading properly. Similarly, different loading flows between 0.25 and 1.5 mL min^{-1} were examined and the best yields were obtained using a flow rate of 0.5 mL min^{-1}.

To eliminate potential polar interferents present in semen samples, deionized water and aqueous solutions of MeOH (2.5 % and 5 % (*v/v*)) were evaluated as SPE washing solutions. 1 mL of an aqueous solution of MeOH (5 % (*v/v*)) was selected as washing condition because no losses were observed when the analyte responses were compared to those obtained in the absence of the washing step.

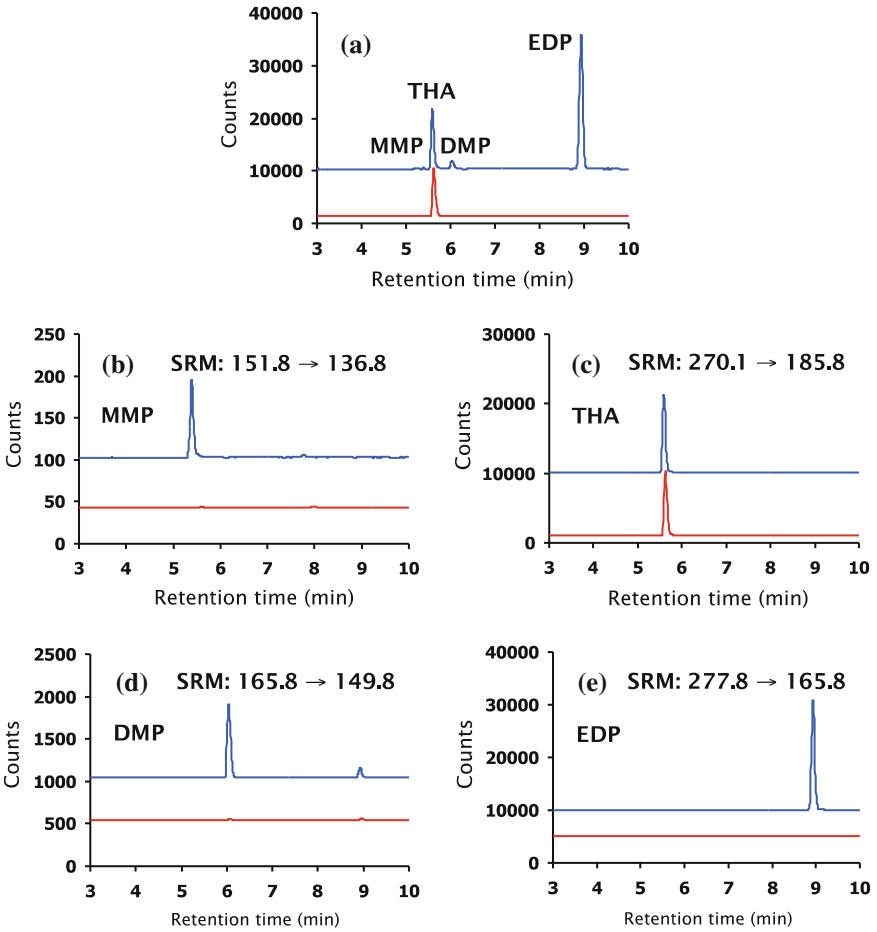

Fig. 8.1 Total ion chromatograms (TIC) in positive ionization mode for each compound of interest (**a**) and selected reaction monitoring (SRM) chromatograms for MMP (**b**), THA (**c**), DMP (**d**) and EDP (**e**) obtained by analyzing a semen sample containing no compounds (Red line) and the same semen sample fortified at a concentration of 50 ng mL^{-1} (Blue line). The internal standard (THA) was included in both types of samples. Experimental conditions are described in Sect. 8.2.3

Analogously, different loading flows were examined and the best yields were obtained using a flow rate of 0.5 mL min^{-1}, as higher flow rates caused the partial elution of the analytes.

The retained analytes were eluted by flowing the LC mobile phase through the SPE sorbent for 4.5 min. Longer elution times allowed the elution of interferents causing ion suppression in the ionization source, while shorter elution times did not provide the quantitative elution of the analytes.

To measure the retention capacity of the analytes in the C18 HD sorbents, breakthrough process was evaluated using a dual cartridge configuration in the

SPE automated platform (Prospekt Technical Note 1996). In this way, two C18 HD cartridges were connected in series to retain in the second cartridge the analytes that have not been retained in the first cartridge during the loading step.

Breakthrough was studied in triplicate analyzing urine samples fortified at three levels of concentration (20, 100 and 500 ng mL^{-1}). Retention efficiency was calculated as the ratio between the analyte response in the first cartridge and the combined response in both cartridges. The experiments conducted under the selected experimental conditions showed that no breakthrough was produced for any analyte at any concentration tested (extraction efficiencies above 95 % in all cases).

Moreover, the cartridges could be reused up to 3 times without causing a significant loss of extraction efficiency (<5 %).

8.3.2 *Validation of the Analytical Method: Study of the Interferences*

To estimate the influence of the matrix sample on the determination of EDP and its metabolites, the responses obtained from a set of standard solutions prepared in a semen sample from a single volunteer containing no analyte (*single semen calibrate*) were compared to those obtained from a set of standard solutions prepared in a pool of semen samples from 5 volunteers containing no analyte (*pooled semen calibrate*) (see Table 8.4).

Table 8.4 Comparison of EDP, DMP and MMP calibrates obtained from single and pooled semen samples

Analyte	Parameter	Calibrate		R (%)[e]	t_{cal}[f]
		Single[d]	Pooled[d]		
MMP	a[a]	0.01 ± 0.07	−0.03 ± 0.05		0.2
	b (mL μg^{-1})[b]	53 ± 2	56 ± 1	95	1.2
	R^{2c}	0.995	0.998		
DMP	a[a]	0.000 ± 0.001	0.0002 ± 0.0006		0.5
	b (mL μg^{-1})[b]	1.97 ± 0.02	1.57 ± 0.02	125	14.9
	R^{2c}	0.9995	0.9996		
EDP	a[a]	0.0000 ± 0.0001	0.0000 ± 0.0001		0.9
	b (mL μg^{-1})[b]	0.164 ± 0.003	0.139 ± 0.004	118	5.9
	R^{2c}	0.998	0.997		

[a] Intercept
[b] Slope
[c] Regression coefficient
[d] Number of points, $N = 5$
[e] Recovery coefficient estimated as the ratio between the slopes ontained using both calibrates
[f] Statistical $t_{tab(0.05,(N1-2)+(N2-2)=6)} = 2.45$ (see Annex III.4)

The slopes and intercepts of both calibrates were statistically compared using a Student's *t* test (see Annex III.4). The intercepts were statistically comparable to zero (at a significance level of 5 %), showing the absence of constant errors. However, the slopes were statistically different at the same level of significance when comparing both calibrates in the cases of DMP and MMP. The t_{cal} statistical values were higher than the t_{tab} values, thus showing a matrix dependency. Therefore, standard addition calibration was used to correct the proportional errors caused by matrix interference.

8.3.2.1 Accuracy

The accuracy was evaluated by applying the SPE-LC–MS/MS method to the analysis of semen samples from different volunteers who had not applied any cosmetic product containing EDP that were fortified with known amounts of EDP, DMP and MMP.

The results obtained for each sample are shown in Table 8.5. The standard deviation was calculated as the error of the extrapolated value in the standard addition curve (Miller and Miller 2005).

The Student's *t*-test confirmed the absence of significant differences between the found concentrations and the fortified concentrations (see Annex III.3), thus showing the accuracy of the proposed methodology.

8.3.2.2 Other Analytical Parameters

Calibration curves (N = 5) exhibited excellent linearity for EDP, DMP and MMP with regression coefficients greater than 0.995 in all cases for a range of concentrations between 20 and 100 ng mL^{-1}.

Table 8.5 Determination of MMP, DMP and EDP in fortified semen samples

Analyte	Parameter	Semen samples				
		1	2	3	4	5
MMP	μ^a	38	47	75	141	235
	$C \pm s^b$	39 ± 4	49 ± 3	78 ± 5	139 ± 6	228 ± 12
	t^c_{cal}	0.79	1.40	1.09	0.87	1.31
DMP	μ^a	39	49	78	146	243
	$C \pm s^b$	37 ± 2	52 ± 3	85 ± 6	151 ± 6	246 ± 13
	t^c_{cal}	1.51	2.12	2.96	1.92	0.69
EDP	μ^a	40	50	80	150	250
	$C \pm s^b$	43 ± 4	52 ± 5	81 ± 3	157 ± 8	247 ± 10
	t^c_{cal}	2.17	1.07	0.51	1.85	0.73

[a] Fortified concentration (ng mL^{-1})
[b] Found concentration (ng mL^{-1}) by standard addition calibration
[c] Statistical $t_{tab(0.05,N-2=3)} = 3.18$

The limits of detection (LOD) and quantification (LOQ) were determined based on the International Conference on Harmonization on validation of analytical procedures (ICH guidelines 2005) (see Table 8.6).

The intra-day variability (repeatability) was assessed by analysing five different semen solutions containing no analytes that were fortified (50 ng mL^{-1}) in the same batch. The inter-day variability (repeatability) was determined by analysing a single semen solution containing no analytes that was fortified (50 ng mL^{-1}) in five different batches.

As can be seen in Table 8.7, the intra-day and inter-day variability, expressed as relative standard deviation (RSD), were in the range from 4.6 to 9.4 %, and from 8.1 to 12.7 %, respectively, depending on the analyte.

8.3.3 Application of the Analytical Method

The described analytical method was applied to the determination of EDP and its metabolites in semen from volunteers which had applied an amount of 13 g of a sunscreen cosmetic product prepared in the laboratory according to an adapted protocol (Jordán and Jordán 1991) containing 8 % of EDP (see Annex II.4). The applied doses are in the normal range of application thickness for sunscreen products (0.5–1 mg cm^{-2}) which, moreover, is well below from the recommended dose to get the labelled value of Sun Protection Factor (SPF) (2 mg cm^{-2}) (Chisvert and Salvador 2007).

In concrete, two different studies were carried out, based on single and repeated sunscreen applications (i.e., in the morning and evening for 4 days, making a total of 8 applications) of the cosmetic product. In the last case, the volunteer was allowed to have a shower per day just before the second daily application. The reason for conducting this study was to simulate a real situation of sunscreen

Table 8.6 Limits of detection (LOD) and limits of quantitation (LOQ) of the developed methodology to determine EDP, DMP and MMP in semen

Sample[a]	MMP		DMP		EDP	
	LOD[b]	LOQ[c]	LOD[b]	LOQ[c]	LOD[b]	LOQ[c]
1	0.5	1.5	0.3	1.0	0.4	1.2
2	0.3	0.9	0.3	1.0	0.6	1.8
3	0.4	1.2	0.4	1.2	0.2	0.6
4	0.4	1.2	0.3	1.0	0.4	1.2
5	0.5	1.5	0.4	1.2	0.4	1.2

[a] Semen samples from different volunteers

[b] Estimated in the semen sample, taking into account the dilution factor during the treatment of the sample, as $\mathbf{3.3}.s_a/b$, where b is the slope of the standard addition calibration curve and s_a is the standard deviation value of the intercept in the calibrate. On-column values, expressed as ng, being the injection volume 100 μL

[c] Estimated in the urine sample as $\mathbf{10}.s_a/b$ (see annotation b)

Table 8.7 Precision parameters for the determination of MMP, DMP and EDP in human semen

Analyte	Parameter	
	Repeatibility (%)[a]	Reproducibility (%)[a]
MMP	6.0	8.1
DMP	4.6	12.5
EDP	9.4	12.7

[a] Expressed as RSD ($N = 5$)

cosmetic product application, which usually involves the application of repeated doses during the sun bathing.

During 8 days from the first or the unique application of the cosmetic product, the whole secreted semen was collected for periods of 24 h into sterile commercially available containers. The total volume of semen daily collected was measured and stored at −20 °C. Considering the total volume of semen, the bioaccumulation process of EDP can be then estimated. Before the first or the unique application of the cosmetic product, semen samples were also collected to check for the absence of EDP and/or its metabolites.

Analogously to the results obtained in urine analysis, EDP was not observed in any of the semen samples that were analyzed, thus showing that EDP follows an extensive biotransformation process in the human body. However, there are clear differences when comparing the studies of semen and urine analysis. Firstly, neither DMP or MMP were detected in semen samples after a single application of the cosmetic product containing EDP. Considering that semen is not considered a proper excretion biofluid, it was not surprising to observe the absence of the metabolites of EDP in semen.

More interesting information could be drawn from study of repeated applications. Thus, the presence of the two metabolites of EDP were detected at different extension (see Fig. 8.2).

Due to lack of enough sample, semen samples were only treated with the ß-glucuronidase solution to determine the total content of the analytes and then, the contribution of both the unconjugated and conjugated content to the total content

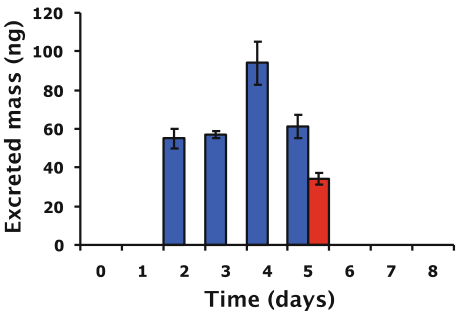

Fig. 8.2 Found amounts of DMP (*blue colour square*) and MMP (*red colour square*) in semen from a volunteer after repeated application of a sunscreen cosmetic product containing EDP. The results are expressed in terms of absolute mass considering the total volume of sample collected

was not determined. However, according to the previous study in urine (see Chap. 7), a low contribution of the unconjugated species to the total content would be expected.

DMP was first detected in the semen sample collected during the period between 24 and 48 h since the start of the study, once the cosmetic product had been applied four times. DMP excretions were measured also in the third, fourth and fifth day after the first cosmetic application. The highest concentration of DMP in human semen was found in the sample collected on the fourth day.

MMP was only detected in the semen sample collected 5 days after the start of the study, corresponding to the samples collected during the 24 h after the eighth application of the cosmetic product. Subsequently, the content of the metabolites of EDP in semen decreased gradually.

Figure 8.3 shows a SRM chromatogram obtained after the application of the SPE-LC-MS/MS methodology to a semen sample collected the fifth day.

As a consequence of the dynamic equilibrium process of bioaccumulation, increasing the incorporation of topically applied EDP to the human body could allow the accumulation of the metabolites of this substance in semen, apart from urine, as an alternative and secondary route of excretion. Taking into account the different levels at which the metabolites of EDP are excreted by semen, more information regarding the mechanism of biotransformation of EDP in humans can be obtained (see Fig. 8.4).

The fact that MMP was only detected in semen after repeated applications of the cosmetic product containing EDP means that initially DMP is formed from EDP by hydrolysis of the 2-ethylhexyl catalyzed by cytochrome P450. Then, this metabolite can be conjugated with glucuronic acid, resulting in the predominant species in urine (DMP-Glu, see Chap. 7) and/or continue the biotransformation process to yield MMP, which may also be conjugated with glucuronic acid, depending on the quantity of EDP absorbed and the studied biofluid.

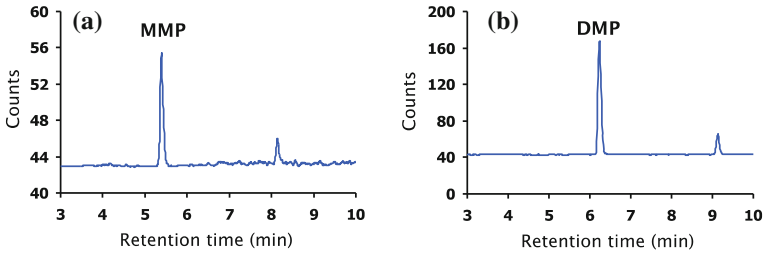

Fig. 8.3 SRM chromatograms for MMP (**a**) and DMP (**b**) obtained by analyzing a semen sample collected the fifth day from a volunteer who had repeatedly applied a cosmetic product containing EDP. Experimental conditions are described in Sect. 8.2.3

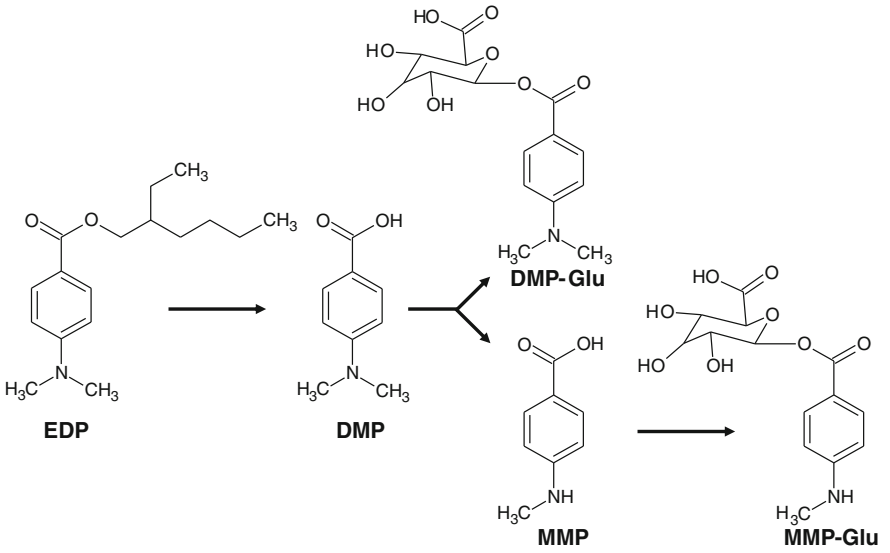

Fig. 8.4 *In vivo* biotransformation mechanism proposed for EDP, thus resulting in DMP, MMP and their respective glucuronide conjugates

8.4 Conclusions

A sensitive analytical method based on automated SPE-LC-MS/MS to determine EDP and its phase I metabolites (DMP and MMP) in human semen with previous ultrasonic assisted enzymatic hydrolysis treatment has been described. The standard addition calibration was used to correct errors caused by matrix interferences. The validation of the method was carried out, thus providing statistically accurate results in the analysis of fortified semen samples.

Furthermore, the proposed methodology has been applied successfully to the analysis of semen samples from volunteers who had applied repeatedly a cosmetic product containing EDP. DMP and MMP were found at different levels in the semen samples, showig evidences of bioaccumulation processes of EDP in humans.

In general, the described analytical methodology could be used to provide more information regarding the toxicology of this substance. Research in the area of male reproductive health should continue in this line, trying to elucidate the magnitude that the effects of EDP and its metabolites can cause in the male reproductive system.

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