Research Article

Determination of methyldibromoglutaronitrile (MDBGN) in skin care products by gaschromatography-mass spectrometry employing an Enhanced Matrix Removal (EMR) Lipid Clean-up

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Running Title MDBGN in skin care products by an EMR Lipid Clean-up

Keywords: complex matrices, EMR—Lipid Clean-up, skin care products, MDBGN, GC-MS analysis.

Abstract

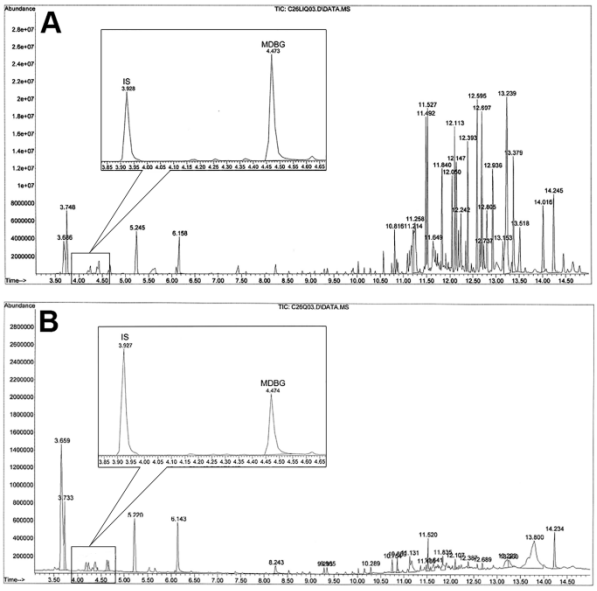
A new application of QuEChERS (Quick, Easy, Cheap, Efficient, Rugged, and Safe) extraction followed by enhanced matrix removal-lipid cleanup and GC-MS analysis is proposed for skin care products. The method was applied to determine methyldibromo glutaronitrile (MDBGN), a preservative frequently used in cosmetic products before being banned for its allergic reactions, so as to unmask its now-prohibited use. The new validated procedure consists in extracting the cosmetic products with acetonitrile, removing the lipid matrix and then water and solid particles from the organic mixture by two dispersive solid-phase extractions (dSPE) in sequence and, lastly, analysing the extracts in GC-MS. Compared to classic liquid-liquid extraction with chloroform, the method has superior features in terms of applicability to cosmetics, ease of use, working times optimization and, above all, reduction of analytically interfering lipidic constituents.

Practical applications

The use of EMR-Lipid dSPE system followed by GC-MS analysis allowed to trace and quantify a minimal amount of a banned preservative, MDBGN, in so-called "complex" matrices, such as cosmetic creams, managing them in a simple and efficient way. Therefore, this system can be proposed for further applications of extractive procedures, advantageously alternative to the classic liquid-liquid extractions, in the field of cosmetics analysis.

The EMR-Lipid dSPE system showed the following advantages: much simpler use, as the system provides tubes already packaged with the clean-up phase, optimization of the working times and noticeable reduction of extraction impurities allowing cleaner extracts to be obtained.

Graphical abstract



A new application of QuEChERS (Quick, Easy, Cheap, Efficient, Rugged, and Safe) extraction followed by enhanced matrix removal-lipid cleanup and GC-MS analysis is proposed for skin care products. Compared to classic liquid-liquid extraction with chloroform (A), the method has superior features in terms of applicability to cosmetics, ease of use, working times optimization and, above all, reduction of analytically interfering lipidic constituents (B).

Introduction

Sample preparation is always necessary when chromatographic analysis is to be applied. The main purpose is to transform the sample into a system suitable for the requested aim: this step becomes fundamental since the primary objective is to obtain a sample that is relatively free of interference, has appropriate chemico-physical characteristics for the analytical method adopted and does not damage the chromatographic columns and/or instruments. Although the main analytical techniques, currently available on the market, are almost fully automated, in most cases the pre-treatment of the sample is still an exclusively manual operation, requiring a lot of time to develop the method. The development of a sample treatment requires careful and early planning especially dealing with complex matrices. A well planned sample preparation procedure should lead to complete analytes recovery in order to improve sensitivity, precision and accuracy of the method, be carried out with a minimum number of steps by reducing the overall time and effort required, and be as automated as possible with the aim of reducing the inaccuracy and precision errors of the analyst. [1-5]

Generally, the separation of analytes of interest from complex matrices is carried out by the application of extractive methods. For liquid (or semi solid) samples, the main extraction techniques used are liquid-liquid extraction (LLE), or solid phase extractions (SPE). In liquid-liquid extractions, extraction solvents such as hexane, acetone, ethyl acetate and dichloromethane may produce complete extraction efficiencies for lipophilic analytes, but may require long and costly steps. Cosmetic products, and in particular creams, are generally semi-solid emulsions of oil in water, consisting of a significant lipid fraction and numerous components with different physical and chemical properties. Interference from lipids is a common problem for all laboratories handling fat complex biological matrices: their presence in a sample could cause remarkable interference, resulting in ion suppression and thus adversely affect sample analysis.

Isolating a lipophilic component in these matrices is a difficult task; in fact, choosing a selective solvent able to extract exclusively the component of interest without dragging the unwanted lipid fraction is almost impossible.

The aim of this work is to propose a new application of sample purification able to produce clean extracts by selectively eliminating lipids from complex matrices without loss of analytes, making sample preparation simple, fast and, above all, reducing the amount of matrix inserts in the instrument. In fact, lipids can accumulate in the column and instrument, reducing the life of the column itself and increasing the frequency of maintenance of the instrument.

The QuEChERS extraction method (Quick, Easy, Cheap, Efficient, Rugged, and Safe) was applied in this work with some modifications to analyse cosmetic products, such as creams and milks, in order to establish whether prohibited methyldibromo glutaronitrile (MDBGN) was present. The analyses were required by a Northern Italy tribunal on commercial cosmetics whose labels declared, due to a labelling mistake according to the producer, the presence of MDBGN and, what's more, without percentage indication. The QuEChERS method was originally developed and utilized for pesticide analysis on samples coming from a large variety of matrices (meat, fish, fruits and vegetables) [6]. Here, in particular, the applicability of Agilent Technologies' Bond Elut Enhanced Matrix Removal-Lipid (EMR-

Lipid) system [7-9] was evaluated on cosmetic creams and milks and the results were compared with those obtained by a classic liquid-liquid extraction in chloroform.
MDBGN (Fig. 1) is a preservative with a potent and clinically significant allergen activity, that has been frequently used since the beginning of the 1980s in industrial and cosmetic products. In 1986, the European Union (EU) Scientific Committee on Cosmetology approved its use in cosmetics at a maximum concentration of 0.1% with the exception of sunscreen products, where the concentration limit was fixed at 0.025%[10].
In 2002 following a substantial increase in cases of eczema, contact dermatitis and allergic phenomena[11], the European Commission (EC), based on the opinion of the Scientific Committee on Cosmetology, proposed to prohibit the use of MDBGN in non-rinse products (leave- On) and to limit its use to only rinsing products, until the actual risk of this compound had been evaluated at normal concentrations of use. The results of numerous surveys led to the development of European Directive 2003/83 / EC, in which the EC has decided to limit the use of MDBGN exclusively to rinsing cosmetic products setting the maximum permissible concentration to 0.1%.[12] However, in recent years, due to a continuous increase in adverse reactions associated with the MDBGN, the EC, with the Community Directive 2007/17 / EC, which was transposed into Italy by a Ministerial Decree of January 2008, forbad the presence of the preservative in all circulating cosmetics in the EU.[13].

Materials and methods

Chemicals and reagents

Acetonitrile HPLC grade (99.9%), methanol (>96%), chloroform (>99,0%), MDBGN, 4-Bromobutyronitrile (IS) (97%), diethoxyacetonitrile and sodium sulfate were purchased from Sigma Aldrich (Milan, Italy). Ethyl 2,3-dibromopropionate was prepared according to a literature method as reported in literature.[14] All reagents were of analytical grade and stored as required by their specifics. Stock standard solutions (1 mg/mL) of MDBGN and IS were prepared in methanol and stored at 4°C for up to a month.
Water (18.2 $\Omega \cdot cm^{-1}$) was prepared by a Milli-Q System (Millipore, Darmstadt, Germany). A dispersive-solid phase extraction kit (EMR—Lipid dSPE, Part No 5982-1010) containing an EMR-Lipid sorbent and Final Polish EMR-Lipid (Part No 5982-0101) containing a mixture of magnesium sulfate and sodium chloride were purchased from Agilent Technologies (Santa Clara, CA).

Cosmetic products

Different cosmetic products were analysed for evaluating the presence of MDBGN:
n. 2 glycolic acid face creams with mallow and aloe, 50 mL;
n. 1 fat and impure skins daily cream, 50 mL;
n. 1 tonic with chamomile and hamamelis distilled water, 500 mL;
n. 1 thermic body massage cream, 1000 mL;
n. 1 cold body massage cream, 1000 mL;
n. 1 detergent milk, 2000 mL;

n. 1 detergent milk with mallow and chamomile extract, 500 mL;
n. 1 detergent milk with mallow and chamomile extract, 250 mL;
n. 1 body massage creams with seaweeds extract, 1000 mL.
The INCI labels of all products contain the caption "*methyldibromo glutaronitrile*" (MDBGN) without indication of substance percentage. All samples were initially screened for MDBGN by applying a liquid-liquid extraction in chloroform. Only the positive samples, in the presence of MDBGN, were analysed by using the EMR-Lipid dSPE.

Sample preparation: liquid – liquid extraction
Aliquots (1 g or 1 mL) of the cosmetic products were accurately weighted into a 15 mL volumetric flask and 200 µL of IS stock solution, 5 mL of water and 5 mL of chloroform were added. The mixture was vigorously shaken for 30 s by vortex, extracted in an automatic rotatory extractor for 5 min and centrifuged at 5500 rpm for 5 min. The organic phase was separated, added with sodium sulfate and centrifuged for 5 min. The extract was transferred to a vial for gas-cromatography analysis. 1µL was injected.

Sample preparation: EMR-Lipid dSPE
Aliquots (1 g) of the cosmetic products were accurately weighted into a 15 mL volumetric flask and 200 µL of IS stock solution and 10 mL of acetonitrile were added. The mixture was vigorously shaken for 30 s by vortex, extracted in an automatic rotatory extractor for 5 min and centrifuged at 6764 g for 5 min. 5 mL of water were added for the activation of EMR-Lipid dSPE 15 mL tube already containing the sorbent for cleanup step, then 5 mL of the previous organic mixture were transferred. The mixture was vigorously shaken for 30 s by vortex, extracted in an automatic rotatory extractor for 5 min and centrifuged at 6764 g for 5 min. 5 mL of extract were transferred to a Final Polish EMR-Lipid tube. The mixture was homogenized during 1 min in vortex, centrifuged and 2 mL of extract were transferred to a vial for gas-chromatography analysis. 1 µL was injected.

Instrumentation and conditions
GC–MS analyses were carried out on a 6890 Series Plus gas chromatograph equipped with an Agilent 7683 autosampler and coupled to a 5973N mass selective detector (Agilent Technologies, Palo Alto, CA, USA). Data were analysed with MSD ChemStation D.03.00 software (Agilent Technologies). Chromatographic separation was carried out on a RXI-5sil-MS capillary column (30 m × 0.25 mm I.D., thickness 0.25 µm; Restek Bellefonte PA, US) and split injection mode (30:1) was used. The GC-MS system was operated under the following conditions: injection temperature: 280°C; interface transfer line: 280°C; ion source: 230°C; initial column temperature: 70°C. The temperature was subsequently increased to 190°C at a rate of 15°C min^{-1}, then to 300°C at a rate of 40°C min^{-1} and held at this temperature for 3.25 min. Helium was used as carrier gas at a flow rate of 1.2 mL/ min. MS analysis was performed in SCAN (40-550 *m*/*z*) and SIM mode with a quadrupole mass detector operated in electron ionization mode, with beam energy of 70 eV. The ions selected for SIM mode acquisition were 106, **66**, 52 for MDBGN and 68, **54** for IS (in bold the quantifier ions).

Validation

Prior to application to real samples, the methods, liquid – liquid extraction and EMR-Lipid dSPE, were tested in a validation protocol scheme following the accepted criteria for bioanalytical method validation. [15] Validation protocol applied in the present study included specificity, precision, accuracy, linearity and limits of detection (LOD) and quantification (LOQ). Standard samples containing different MDBGN amounts were prepared by adding suitable amounts of standard stock solutions of MDBGN to 1 g of “fat and impure skins daily creams” which were negative at the presence of the MDBGN. The standard samples were then treated as reported in the "sample preparation" section.

The specificity was assessed by extracting control blank samples in each validation run. The lack of interfering peaks at the same analyte retention times was considered as an acceptable selectivity.

Validation parameters for precision and accuracy were calculated using different replicates of samples in different working days. Accuracy was expressed as the percent recovery (%REC), while precision was measured as coefficient of variation (CV%). A CV% below 10% was considered suitable.

Calibration curves were calculated by plotting peak area MDBGN/ area IS versus the total amount (µg) of MDBGN added in the range 50-800 (50, 100, 250, 500, 800) total µg of analyte in 1 g of blank matrix.

The LOD, defined as the lowest concentration of analyte that can be clearly detected, is estimated as three times the signal to noise ratio. LOQ is the lowest concentration that met a signal-to-noise ratio of at least 10.

Results and Discussion

Method development

During the development of the method for the determination of MDBGN, different compounds were considered for the selection of the internal standard. In particular, the following molecules have been studied: 4-bromobutyronitrile (A), ethyl 2,3-dibromopropionate (B), and diethoxyacetonitrile (C) (Fig. 1). Diethoxyacetonitrile was discarded because it was not suitable for use in GC/MS under the adopted conditions due to a too short retention time. Ethyl 2,3-dibromopropionate initially seemed to be the most suitable molecule, but it was subsequently discarded owing to poor solubility in the solvents used in MDBGN extraction procedures, leading to very high coefficient of variation values (CV%) and therefore inadequate for the purpose. On the other hand, 4-bromobutyronitrile was found to be relevant as IS for two reasons: first for its chemical structure characterized by the presence of bromine atom and nitrile group as well as in the MDBGN molecule, and secondly for its chromatographic behaviour which was very similar to the molecule of interest (Fig. 2). Moreover the reproducibility and recovery values obtained were acceptable.

A study on the injection system (split/splitless) and on injector temperature was also necessary. The most suitable injection system was the split method under the conditions described in the method. The splitless method led to excellent responses in terms of absolute area for the MDBGN molecule, but to a loss in resolution for the IS molecule.

Therefore, it was decided to work in split mode since sensitivity was good enough for the purposes of this work.
The working temperature of the injector was evaluated in a temperature range of 150 to 290°C by analysing a 100 µg/mL MDBGN solution. The chosen working temperature was 220°C because at this value the highest response and resolution of the MDBGN peak were obtained.

Validation parameters

The results obtained from the validation study fulfilled the expectations for both methods. Initially, all tested products were subjected to liquid-liquid extraction as reported and no interfering peaks appeared at the retention time of the MDBGN and IS molecule. The specificity was evaluated also for the EMR-Lipid dSPE technique, confirming the expected data.
The precision and accuracy results are reported in Table 1 for both methods: accuracy values never above 10% (expected value) and recovery values around 100% were obtained. These results were attained by testing standard samples with 200 µg/g MDBGN concentration in replicate.
The linearity was proven according to the regression line by the method of least squares and expressed by the coefficient of determination (R^2). Five-point matrix-matched calibration curves were evaluated by spiking increasing amounts of the analyte in blank matrix samples. Calibration curves were obtained by plotting the ratio between the peak area of the quantifier ion of the analyte and the peak area of the quantifier ion of the internal standard versus the corresponding concentrations of the analytes in concentration range between 50 and 800 µg/g of MDBGN. We observed linearity in the whole range. The values of the correlation factors R^2 of the calibration curves were higher than 0.99. The LOD and LOQ values obtained were respectively 10 and 50 µg/g suitable for the purposes of the work (Table 2).

Analysis of skin care products
The EMR-Lipid dSPE system was first applied in our laboratory as an effective lipophilic fraction removal matrix system. As mentioned before, the presence of lipids (fatty acids, phospholipids, cholesterol, etc...) may cause significant interference and adversely affect the sample analysis. In fact, lipids can accumulate in the column and instrument, reducing the life of the column itself and increasing the frequency of instrument maintenance. This new matrix purification system was applied to cosmetic creams evaluating the applicability of the method in terms of ease of application, reduced consumption, speed of execution and cleaning of extracts compared to a classic liquid-liquid extraction.
This system is available on the market in a ready-to-use form. It consists of two falcon-type tubes: the first tube contains a solid extraction phase in which the lipid and fat functions are mainly retained, the second tube contains a mixture of magnesium sulphate and sodium chloride for an effective removal of water and dispersed solid particles. The tubes are characterized by caps of different colours that identify the contents of the tubes, greatly limiting the possibility of error by the operator. The advantage of having many samples in

pre-prepared tubes is that it is possible to prepare many samples at the same time with low error chance by operators and, above all, to make the preparation systematic. In addition, relatively simple laboratory equipment is required: an extractor and a centrifuge. For our work, the extract obtained did not need to be concentrated, but this system allows to do that if necessary.

MDBGN was detected only in the two samples of glycolic acid face creams with mallow and aloe. The concentration of MDBGN (Table 3) determined by the two different extraction procedures were comparable: 0.022% by liquid-liquid extraction and 0.019% by EMR-Lipid dSPE system. Therefore, both extraction methods can be considered effective. Nevertheless, the EMR-Lipid dSPE system showed the following advantages: much simpler use, as the system provides tubes already packaged with the cleanup phase, optimization of the working times and noticeable reduction of extraction impurities allowing cleaner extracts to be obtained. From the chromatograms reported in Fig. 3, it is evident that the extract obtained after LLE contains a lot of impurities related to the matrix (peaks from 10.50 to 14.50 min, Fig. 3A), that interfere with the analysis and cause damage to the chromatographic column. On the other hand, the extract obtained after the application of the EMR-Lipid dSPE method is considerably cleaner (Fig. 3B). In Fig. 3A and 3B the Full Scan acquisition mode is reported. In the insets, the SIM (single ion monitoring) acquisition is shown to evidence the presence of the analyte and the IS in the extract.

With regard to the tribunal's request and to the potential allergenic activity of the analysed cream samples, it must be pointed out that, although the found MDBGN concentration was very low, this glycolic acid face cream is classified as a cosmetic product without rinsing (leave-on product) suitable for daily application and that MDBGN was allowed at 0.025% concentration in non-rinse product before 2002, completely banned from these products after 2002 and prohibited in cosmetic products of any kind in 2007. Such prescriptions do not leave room for any objections regarding real likelihood of health damage at this MDBGN concentration and at the recommended frequency of product application.

Conclusion

The use of EMR-Lipid dSPE system followed by GC-MS analysis allowed to trace and quantify a minimal amount of a banned preservative, MDBGN, in so-called "complex" matrices, such as cosmetic creams, managing them in a simple and efficient way. Therefore, this system can be proposed for further applications of extractive procedures, advantageously alternative to the classic liquid-liquid extractions, in the field of cosmetics analysis.

The authors have declared no conflict of interest.

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Table 1. Validation parameters: intra-interday precision and accuracy of MDBGN in cream samples.

Method	Precision (CV%) n=6		Accuracy (REC%) *n=5*
	intraday	interday	
EMR-Lipid	7.2	7.7	100.0
LLE	8.0	8.2	103.2

Table 2. Validation parameters: calibration curve parameters, LOD and LOQ of MDBGN.

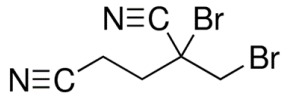
Method	Range (µg tot) n= 5	Linearity equation	Correlation Coefficient (R^2)	LOD (µg/g)	LOQ (µg/g)
EMR-Lipid	50-800	y = 0.0051x – 0.1367	0.9985	10	50
LLE	50-800	y = 0.0053x – 0.0417	1.0000	10	50

Table 3 Concentration and percentage of MDBGN in seized cosmetic products.

	EMR-Lipid		LLE	
Cosmetic product	MDBGN (µg/g) (*n=6*)	% MDBGN	MDBGN (µg/g) (*n=6*)	% MDBGN
Glycolic acid face cream	187.8 ± 11.5	0.019	224.6 ± 17.3	0.022
Fat and impure skins daily cream	n.d.	n.d.	n.d.	n.d.
Tonic	-	-	n.d.	n.d.
Thermic body massage cream	-	-	n.d.	n.d.
Cold body massage cream	-	-	n.d.	n.d.
Detergent milk	-	-	n.d.	n.d.
Body massage cream with seaweeds extract	-	-	n.d.	n.d.

n.d. = not detected

Fig. 1 The chemical structure of methyldibromo glutaronitrile (MDBGN, A), 4-bromobutyronitrile (B), ethyl 2,3-dibromopropionate (C), and diethoxyacetonitrile (D).



A

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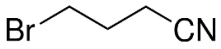

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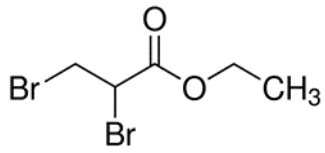
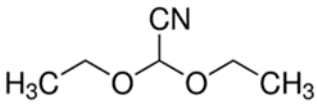


C

M.W. = 259.92



D

M.W. = 129.16

Fig. 2 GC-MS chromatogram in selected ion monitoring (SIM) and mass spectrum of a standard solution of IS (t_R = 3.928 min) and MDBGN 1mg/mL (t_R = 4.474 min).

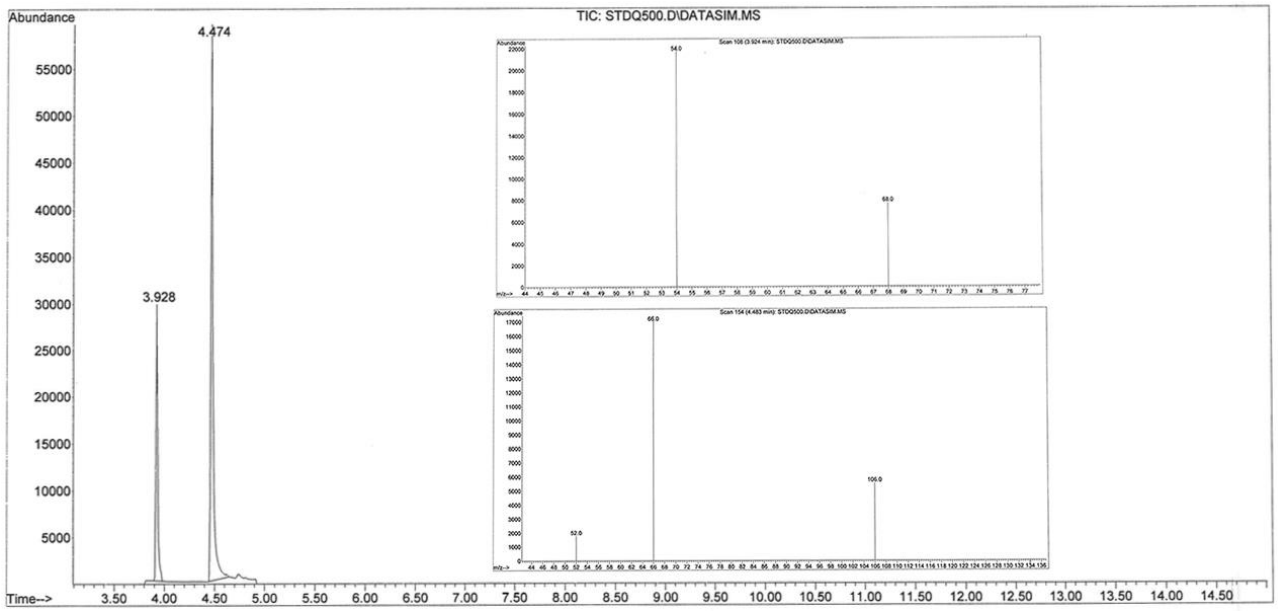


Fig. 3: GC-MS chromatograms in Full Scan mode of a sample of face cream extract with LLE (A) and EMR-Lipid dSPE (B). In the inset the SIM acquisition is reported.