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Rapid determination of furanocoumarins in creams and pomades using SPE and GC

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Abstract

A solid-phase extraction and chromatography-flame ionization detection (GC-FID) method has been developed for the routine analysis of psoralen, bergapten, isopimpinellin and pimpinellin in creams and pomades employed in Brazil for the treatment of vitiligo. The calibration curve for psoralen was linear in the range 10–100 μg ml^{-1}, for bergapten 5–90 μg ml^{-1}, for pimpinellin 10–90 μg ml^{-1} and for isopimpinellin 5–100 μg ml^{-1}. The best recoveries of the furanocoumarins in the creams analysed were 94–97%, whereas in the pomades, recoveries were 94–96%. The R.S.D. of the quantitative analysis of the furanocoumarins in the products analyses were within 5%.

Keywords: Gas chromatography; Furanocoumarins; Psoralens; Vitiligo; Creams; Pomades

1. Introduction

Furanocoumarins or psoralens are well known photoreactive compounds [1], psoralen and bergapten are used in some pharmaceutical and cosmetic products because of their UV-light absorbing properties [2]. Furanocoumarins are also increasingly used in dermatology for the photochemotherapy of diseases such as vitiligo, psoriasis, mycosis fungoides, atopic eczema and alopecia areata among others [3,4]. The biological activity of these compounds is attributable to their ability to intercalate into DNA where they form mono- and di-adducts in the presence of long-wave UV light [5]. Linear furanocoumarins cause phototoxicity [6] and isopimpinellin is considered the least phototoxic [7]. Furthermore, the use of these furanocoumarins in medicine has been associated with a higher incidence of skin cancer [8,9]. Several studies have demonstrated that the furanocoumarins are carcinogenic, mutagenic and photodermatitic [10,11].

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The psoralens are currently employed in dermatology (orally or topically), associated with Ultraviolet A (UVA) irradiation. The combination of these previous elements is known as PUVA therapy [12].

A number of methods has been described for the analysis of furanocoumarins, among them high performance liquid chromatography (HPLC) and capillary gas chromatography (GC). This last technique has been shown to be a very efficient system for separating such mixtures due to its speed and resolution [13–16].

Many popular phytomedicines and phytocosmetics are used in Brazil against skin diseases and suntan preparations. However, most of them are not submitted to any quality control, leading to adulterations that impose serious risks to public health. For instance, the present work started because a patient employed cream 2 causing intense photodermatitis. The patient was self-medicated and the flask had no information on the cream composition. Creams 1 and 3–8 employed in the studies also had no indications of their composition. The pomades had indications of the presence of bergapten, without exact dosages.

There are not previous descriptions in the literature on sample preparation and GC-DIC analysis of furanocoumarins present in phytomedicines (creams and pomades) employed in the treatment of vitiligo in Brazil.

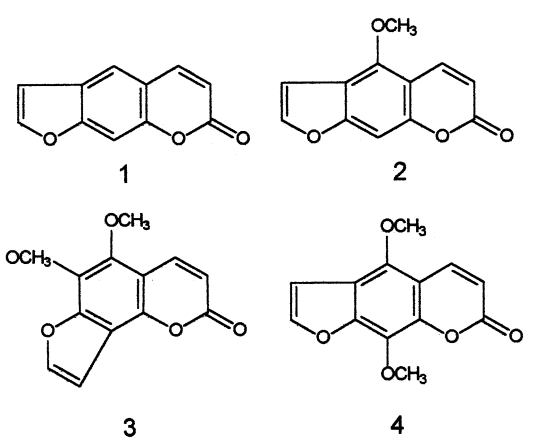

Fig. 1. Chemical structure of the compounds detected by GC-FID.

In the present work, we describe a method for sample preparation and GC-FID analyses of furanocoumarins (psoralen (**1**), bergapten (**2**), pimpinellin (**3**) and isopimpinellin (**4**) (Fig. 1) present in creams and pomades.

2. Experimental

2.1. Products

Products (eight creams and three pomades) were used for the development of the method.

2.2. Chemicals

Spectroscopy-grade methanol and chloroform were purchased from Merck (Darmstadt, Germany). Furanocoumarins standards were obtained from a collection in our laboratory. Stock mixtures of furanocoumarins standards were made up from the individual solutions in chloroform and used as external standards and directly analysed by GC-FID.

2.3. Apparatus

The samples were analysed by GC-FID. These analyses were performed in a VARIAN 3400 gas chromatograph equipped with a capillary fused silica LM-5 (15 m × 0.2 mm i.d., film thickness 0.5 μm) and with a flame ionization detector (FID). H_2 was used as carrier gas at a flow rate 0.8 ml min^{-1} and the injection split ratio was 1:20. The injection temperature was 280°C. Column temperature was programmed from 150 to 240°C with a linear increase of 10°C min^{-1}, then 240–280°C with a linear increase of 5°C min^{-1} and was then held for 15 min. The detector temperature was 280°C. Samples of 1 μl were injected with a 10-μl Hamilton syringe.

2.4. Sample preparation

2.4.1. Extraction creams and pomades

The samples (0.1 g) were extracted either with 10 ml of chloroform or with 10 ml methanol in a sonic bath for 20 min and the solutions were

filtered with filter paper. The solvents were evaporated under a nitrogen stream.

2.4.2. Clean-up 1

Each sample extracted above was redissolved in 1 ml of chloroform and fractionated on cartridges (Sep-pak Silica, Millipore, 690 mg, 55–105 μm), which were previously conditioned with 20 ml of chloroform and sequentially eluted with 5 ml chloroform (first fraction), 10 ml chloroform/methanol (90:10 v/v) (second fraction, furanocoumarins), 10 ml chloroform/methanol (1:1 v/v) (third fraction) and 10 ml methanol (fourth fraction). The four fractions were evaporated to dryness in a stream of nitrogen. Each residue was dissolved and a solution diluted in chloroform in a 5-ml volumetric flask, filtered through a 0.45-μm Millex filter and analysed directly by GC-FID.

2.4.3. Clean-up 2

Each sample extracted above was redissolved in 1 ml of methanol in a sonic bath for 2 min and fractionated on cartridges (Sep-pak C_{18}, Millipore, 360 mg, 55–105 μm), which were previously conditioned with 20 ml of methanol and sequentially eluted with 2 ml methanol (first fraction), 10 ml methanol/chloroform (80:20 v/v) (second fraction, furanocoumarins), 10 ml methanol/chloroform (1:1 v/v) (third fraction) and 10 ml chloroform (fourth fraction). The four fractions were evaporated to dryness in a stream of nitrogen. Each residue was dissolved and a solution diluted in chloroform in a 5-ml volumetric flask, filtered through a 0.45-μm Millex filter and analysed directly by GC-FID.

2.4.4. Recovery experiments

Recovery was determined with spiked samples of creams and pomades with solutions of low, medium and high concentration levels of each furanocoumarin (psoralen, bergapten, pimpinellin and isopimpinellin). The spiked samples were submitted to the same procedure, described above.

2.4.5. Calibration curves

Estimation of the content of psoralen, bergapten, pimpinellin and isopimpinellin in creams and pomades was carried out by external calibration. The compounds were dissolved separately in analytical grade chloroform in order to obtain the stock solutions which were appropriately diluted to concentrations ranging from 1 to 100 μg ml^{-1} of compounds. Aliquots (1.0 μl) for 10 dilutions of each standard were analysed by GC-FID.

3. Results and discussion

The use of these furanocoumarins in medicine for the treatment of vitiligo has been associated with a higher incidence of skin cancer [8,9]. There were indications that some creams and pomades sold in Brazil for the treatment of vitiligo contained those furanocoumarins. Thus, they were selected for the development of the analytical method in the present study. Samples were extracted either with chloroform or methanol in order to obtain 0.2% solutions. A number of preliminary GC-experiments, employing creams and pomades solutions, were performed to establish optimal conditions for GC-FID analysis of furanocoumarins.

GC analysis showed baseline separation for the compounds of interest, which could be analysed in a satisfactory time interval of less than 8 min in the case of the standard mixture [17]. The relatively short time for psoralen, bergapten, pimpinellin and isopimpinellin allows the analysis of a large number of samples.

The identification of furanocoumarins in the creams and pomades was performed by comparison of their retention time with those of authentic samples by co-injection of authentic standards.

The results showed that extraction with methanol afforded better chromatographic results, since chloroform also extracted many other substances, leading to lower selectivity and peaks with retention times close to those of the furanocoumarins, both for clean-up 1 and clean-up 2.

No changes in furanocoumarins (psoralen, bergapten, pimpinellin and isopimpinellin) concentrations were detected in working standard solutions after 1 month of storage at 4°C and at −20°C for at least 6 months. The extracts from samples were stable for at least 24 h at room

Table 1
Regression data for calibration lines for quantitative determination of psoralen, bergapten, pimpinellin and isopimpinellin by GC-FID[a]

Substance	Psoralen	Bergapten	Pimpinellin	Isopimpinellin
Linear range (μg ml^{-1})	10–100	5–90	10–90	5–100
b	232.92	246.84	275.99	239.08
a	12.09	−35.09	−109.45	25.05
Sa	78.22	5.10	241.12	43.43
Sb	1.11	0.67	4.20	0.69
R	0.9999	0.9999	0.9996	0.9999
n	7	6	5	9

[a] *b*, slope; *a*, intercept, *Sb*, S.D. of the slope; *Sa*, S.D. of the intercept; *R*, correlation coefficient; *n*, data number.

temperature. Investigations on the stability of the standards during clean-up 1 revealed a 1% decrease in the concentrations after the procedure and for clean-up 2, a 4% decrease occurred.

The calibration curves were determined by linear regression. The calibration curve for psoralen was linear in the range 10–100 μg ml^{-1}, for bergapten 5–90 μg ml^{-1}, for pimpinellin 10–90 μg ml^{-1} and for isopimpinellin 5–100 μg ml^{-1} (Table 1). Average standard errors for the peak areas of replicate injections ($n = 5$) were less than 5% showing good repeatability of the calibration curve.

The limit of detection and the quantification limit were determined, employing the method of the IUPAC [18]. The limits of detection were for psoralen 1.3 μg ml^{-1}, bergapten 0.6 μg ml^{-1}, pimpinellin 2.9 μg ml^{-1} and isopimpinellin 1.0 μg ml^{-1}. The limits of quantification (LOQ) were for psoralen 4.3 μg ml^{-1}, bergapten 2.0 μg ml^{-1}, pimpinellin 9.7 μg ml^{-1} and isopimpinellin 3.0 μg ml^{-1}.

The recovery experiments with the creams gave 94.9 (±1.3)% for psoralen, 94.0 (±2.3)% for bergapten, 96.6 (±1.8)% for isopimpinellin and 97.0 (±3.4)% for pimpinellin in clean-up 1 and 78.2 (±4.3)% for psoralen, 80.0 (±4.1)% for bergapten, 89.0 (±3.7)% for isopimpinellin and 88.4 (±4.4)% for pimpinellin in clean-up 2. The recovery experiments with the pomades gave 95.2 (±2.4)% for psoralen, 94.7 (±3.3)% for bergapten, 95.1 (±4.8)% for isopimpinellin and 95.3 (±3.7)% for pimpinellin with clean-up 1 and 80.9 (±3.5)% for psoralen, 71.8 (±4.3)% for bergapten, 82.6 (±3.6)% for isopimpinellin and 77.6 (±3.4)% for pimpinellin in clean-up 2. The results showed that the procedure used in clean-up 1 is good both for the creams and for the pomades, since the loss of furanocoumarins by volatilization due to the nitrogen stream and due to adsorption into the cartridges is negligible and allows satisfactory GC quantification of these compounds.

The accuracy of the assay method was evaluated by calculating the mean percent differences between theoretical and measured values. The mean value should be within ±5% of the actual value.

The accuracy values in intra-day variation studies at low, medium and high concentrations of psoralen, bergapten, pimpinellin and isopimpinellin in creams and pomades were within acceptable limits (Tables 2 and 3).

The precision of a method is expressed as the percentage coefficient of variation (%CV) of the replicate measurements. To be acceptable, the %CV should be within ±5% at all concentrations. In this study, the precision of the method was tested for both intra-day and inter-day reproducibility in creams and pomades.

The intra-day variability of the assay method was determined by the repeated analysis of the quality control samples ($n = 5$) at low, medium and high concentrations on the sample day. The results are shown in Tables 4 and 5. These data indicate that the assay method is reproducible within the same day.

Table 2
Accuracy of the GC-FID method for determination of psoralen, bergapten, pimpinellin and isopimpinellin in cream samples ($n = 5$)[a]

C. added ($\mu g\ ml^{-1}$)	Psoralen		Bergapten		Pimpinellin		Isopimpinellin	
	C. found ($\mu g\ ml^{-1}$)	Accuracy (%)	C. found ($\mu g\ ml^{-1}$)	Accuracy (%)	C. found ($\mu g\ ml^{-1}$)	Accuracy (%)	C. found ($\mu g\ ml^{-1}$)	Accuracy (%)
10	9.9 ± 0.41	4.08	9.9 ± 0.43	4.22	9.9 ± 0.31	3.34	9.9 ± 0.29	3.56
50	49 ± 1.63	4.05	49 ± 1.15	3.06	49 ± 1.13	3.16	49 ± 1.73	3.76
90	89 ± 3.01	3.16	89 ± 2.23	2.57	89 ± 2.49	2.89	89 ± 2.07	2.53

[a] Values are mean $\pm$ S.D.

Table 3
Accuracy of the GC-FID method for determination of psoralen, bergapten, pimpinellin and isopimpinellin in pomade samples ($n = 5$)[a]

C. added (μg ml^{-1})	Psoralen		Bergapten		Pimpinellin		Isopimpinellin	
	C. found (μg ml^{-1})	Accuracy (%)	C. found (μg ml^{-1})	Accuracy (%)	C. found (μg ml^{-1})	Accuracy (%)	C. found (μg ml^{-1})	Accuracy (%)
10	9.9 ± 0.29	3.36	9.9 ± 0.43	4.02	9.9 ± 0.47	4.26	9.9 ± 0.45	4.10
50	49 ± 1.95	3.56	49 ± 1.67	3.46	49 ± 1.89	3.96	49 ± 1.93	4.02
90	89 ± 2.01	2.59	89 ± 2.25	2.48	89 ± 2.17	2.62	89 ± 2.33	2.64

[a] Values are mean ± S.D.

Table 4
Intra-day variability of the GC-FID method for determination of psoralen, bergapten, pimpinellin and isopimpinellin in cream samples ($n = 5$)[a]

C. added (μg ml^{-1})	Psoralen		Bergapten		Pimpinellin		Isopimpinellin	
	C. found (μg ml^{-1})	Accuracy (%)	C. found (μg ml^{-1})	Accuracy (%)	C. found (μg ml^{-1})	Accuracy (%)	C. found (μg ml^{-1})	Accuracy (%)
10	9.9 ± 0.41	2.93	9.9 ± 0.43	4.34	9.9 ± 0.31	4.75	9.9 ± 0.29	2.93
50	49 ± 1.63	3.98	49 ± 1.15	3.41	49 ± 1.13	3.86	49 ± 1.73	3.53
90	89 ± 2.01	2.26	89 ± 2.23	2.53	89 ± 2.49	2.44	89 ± 2.07	2.33

[a] Values are mean ± S.D.

Table 5
Intra-day variability of the GC-FID method for determination of psoralen, bergapten, pimpinellin and isopimpinellin in pomade samples ($n = 5$)[a]

C. added (μg ml^{-1})	Psoralen		Bergapten		Pimpinellin		Isopimpinellin	
	C. found (μg ml^{-1})	Accuracy (%)	C. found (μg ml^{-1})	Accuracy (%)	C. found (μg ml^{-1})	Accuracy (%)	C. found (μg ml^{-1})	Accuracy (%)
10	9.9 ± 0.29	2.93	9.9 ± 0.43	4.34	9.9 ± 0.47	4.75	9.9 ± 0.45	4.55
50	49 ± 1.95	3.98	49 ± 1.67	3.41	49 ± 1.89	3.86	49 ± 1.93	3.94
90	89 ± 2.01	2.26	89 ± 2.25	2.53	89 ± 2.17	2.44	89 ± 2.33	2.62

[a] Values are mean ± S.D.

Table 6
Inter-day variability of the GC-FID method for determination of psoralen, bergapten, pimpinellin and isopimpinellin in cream samples ($n = 5$)[a]

C. added (μg ml^{-1})	Psoralen		Bergapten		Pimpinellin		Isopimpinellin	
	C. found (μg ml^{-1})	Accuracy (%)	C. found (μg ml^{-1})	Accuracy (%)	C. found (μg ml^{-1})	Accuracy (%)	C. found (μg ml^{-1})	Accuracy (%)
10	9.9 ± 0.49	4.95	9.9 ± 0.43	4.34	9.8 ± 0.40	4.08	9.9 ± 0.38	3.84
50	49 ± 1.95	3.98	49 ± 1.27	2.59	49 ± 1.19	2.43	49 ± 1.87	3.82
90	89 ± 3.87	4.35	89 ± 2.94	3.30	89 ± 2.69	3.02	89 ± 3.31	3.72

[a] Values are mean ± S.D.

Table 7
Inter-day variability of the GC-FID method for determination of psoralen, bergapten, pimpinellin and isopimpinellin in pomade samples ($n = 5$)[a]

C. added (μg ml^{-1})	Psoralen		Bergapten		Pimpinellin		Isopimpinellin	
	C. found (μg ml^{-1})	Accuracy (%)	C. found (μg ml^{-1})	Accuracy (%)	C. found (μg ml^{-1})	Accuracy (%)	C. found (μg ml^{-1})	Accuracy (%)
10	9.9 ± 0.39	3.94	9.8 ± 0.48	4.85	9.8 ± 0.47	4.80	9.9 ± 0.49	4.95
50	49 ± 2.35	4.80	49 ± 2.01	4.10	49 ± 1.89	3.86	49 ± 2.13	4.35
90	89 ± 2.81	3.16	89 ± 2.75	3.09	89 ± 2.37	2.66	89 ± 2.98	3.35

[a] Values are mean ± S.D.

Table 8
Contents (μg $g^{-1} \pm$ R.S.D.) of furanocoumarins in creams and pomades using clean-up 1 and gas chromatography[a]

Sample	Psoralen	Bergapten	Pimpinellin	Isopimpinellin
Cream 1	495 ± 3.9	740 ± 4.1	–	–
Cream 2	530 ± 2.3	341 ± 2.9	–	–
Cream 3	720 ± 3.7	382 ± 4.9	–	–
Cream 4	–	610 ± 3.6	–	–
Cream 5	510 ± 4.3	590 ± 2.8	–	–
Cream 6	–	435 ± 3.0	–	–
Cream 7	–	566 ± 4.5	–	–
Cream 8	–	–	575 ± 4.9	300 ± 1.3
Pomade 1	544 ± 3.7	678 ± 4.5	–	–
Pomade 2	–	580 ± 2.1	–	–
Pomade 3	–	563 ± 1.8	–	–

[a] Average of five determinations each: S.D. < 5%; –, not detected.

The inter-day variability of the assay method was determined by the repeated analysis of the quality control samples ($n = 5$) at low, medium and high concentrations on three different days. The quality control samples were prepared on the same day at each concentration, and then divided into aliquots that were stored at −20°C until analysis. The results are shown in Tables 6 and 7. These data indicate that the assay method is reproducible on the different days.

Tables 8 and 9 show the contents of the furanocoumarins in each cream and pomade analysed using the optimized conditions (extraction with methanol and clean-up 1) by GC-FID (Table 8) and HPLC-UV [8] (Table 9). We observed qualitative and quantitative discrepancies among the samples (Table 8). No discrepancies in relation to dosage by GC-FID and HPLC-UV were observed in Tables 8 and 9.

Literature reports that the concentration of furanocoumarins in topic preparations used against vitiligo must be between 0.001 and 1% [19] and 1% with possible dilutions of 1:1000 and 1:10 000 [20]. In our study, we have found concentrations between 0.030 and 0.074%. Therefore, the analysed samples were all in the range of the therapeutic doses. A representative chromatogram from the analysis is shown in Fig. 2.

The GC-FID assay method present here is rapid, sensitive and robust and can be applied to

Table 9
Contents (μg $g^{-1} \pm$ R.S.D.) of furanocoumarins in creams and pomades using clean-up 1 and HPLC [8][a]

Sample	Psoralen	Bergapten	Pimpinellin	Isopimpinellin
Cream 1	473 ± 2.7	710 ± 3.2	–	–
Cream 2	514 ± 3.6	320 ± 4.1	–	–
Cream 3	678 ± 3.2	372 ± 4.9	–	–
Cream 4	–	595 ± 3.4	–	–
Cream 5	498 ± 4.8	575 ± 3.5	–	–
Cream 6	–	423 ± 3.9	–	–
Cream 7	–	547 ± 4.0	–	–
Cream 8	–	–	ND	290 ± 3.2
Pomade 1	520 ± 3.3	640 ± 3.1	–	–
Pomade 2	–	560 ± 4.4	–	–
Pomade 3	–	550 ± 2.9	–	–

[a] Average of five determinations each: S.D. < 5%; –, not detected; ND, not determined.

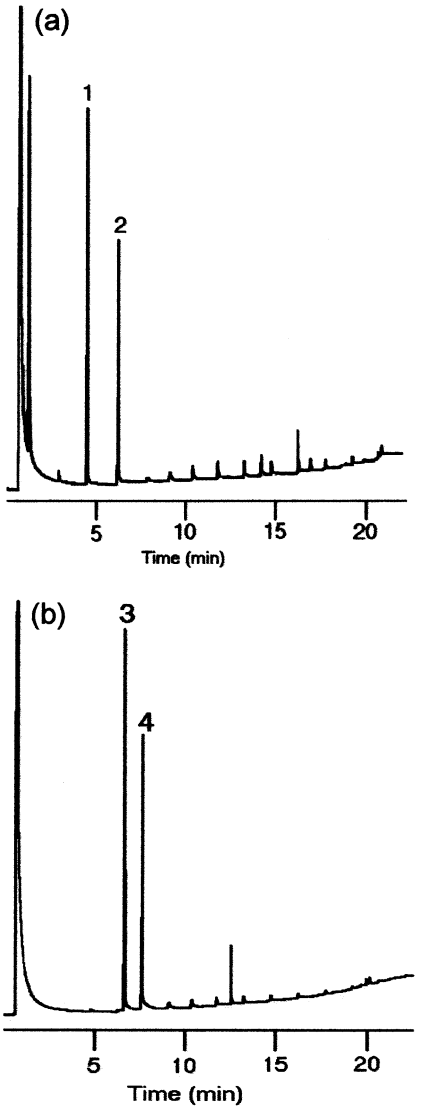

Fig. 2. (a) Representative chromatogram of the cream 2: 1, psoralen; 2, bergapten; (b) Representative chromatogram of the cream 8: 3, pimpinellin; 4, isopimpinellin.

the determination of furanocoumarins in routine analysis of creams, pomades and other lipophilic phytocosmetics, providing a method for their quality control.

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