Version 8 (12/04/2013)

Quantification of Furocoumarins

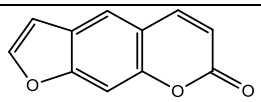
HPLC/DAD Procedure

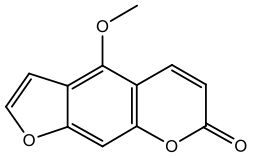
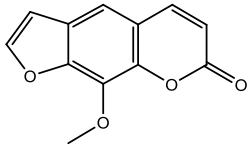
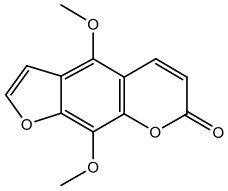
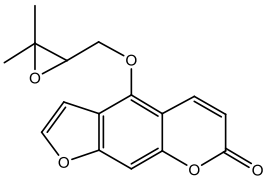
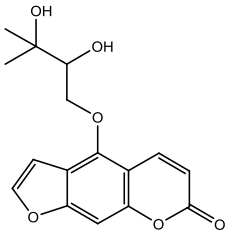
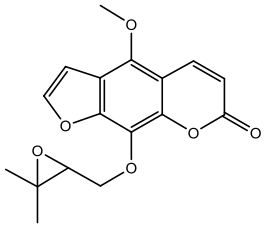
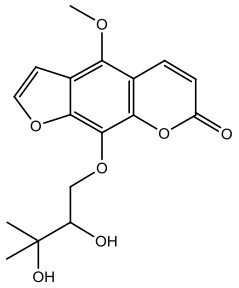
Scope

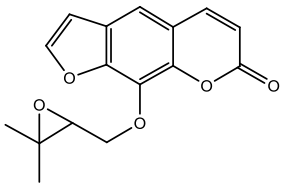
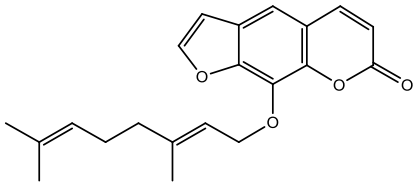
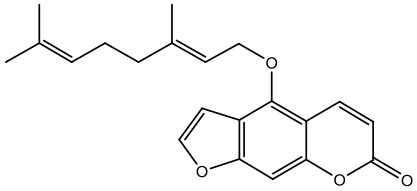
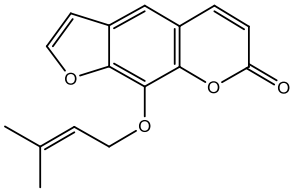
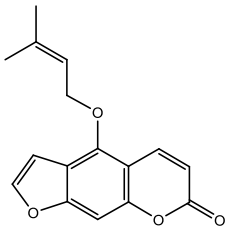
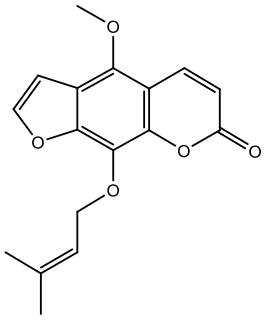
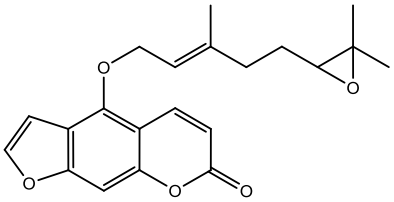
The European Cosmetics Directive 76/768/EEC, which is about to be adopted in the European Cosmetic Regulation, in Annex II (entry 358) contains a limit on the presence and use of the photosensitizing furocoumarins (FCs) in cosmetic products. It is stated that FCs (e.g. xanthotoxin or bergapten) are prohibited, except when they derive directly from the natural materials used. In both sun-protection and bronzing products, those FCs resulting from natural materials shall be below 1 mg/kg. The same restriction applies under the ASEAN Cosmetics Directive.

In a more recent proposal by the Scientific Advisory Committee to the European Commission (SCCNFP/0392/00 of 25 September, 2000 titled: "Opinion on the initial list of perfumery materials which must not form part of cosmetic products except subject to restrictions and conditions laid down"), this 1 mg/kg limit is proposed to be generalized to all cosmetic products, but to date, this opinion has not been adopted in any regulatory proposal.

The capabilities and limits of an HPLC-UV procedure [1] have been investigated by the Analytical Working group of IFRA and published within a scientific publication [2]. Based on these results, this method describes the quantification of 15 furocoumarins, namely:

	Furocoumarin	CAS-RN	Structure
1	Psoralen	000066-97-7	

2	Bergapten	000484-20-8	
3	Xanthotoxin	000298-81-7	
4	Isopimpinellin	000482-27-9	
5	Oxypeucedanin	000737-52-0	
6	Oxypeucedanin hydrate	002643-85-8	
7	Byakangelicol	061046-59-1	
8	Byakangelicin	000482-25-7	

9	Heraclenin	035740-18-2	
10	8-Geranyloxypsoralen	071612-25-4	
11	Bergamottin	000482-46-2	
12	Imperatorin	000482-44-0	
13	Isoimperatorin	000482-45-1	
14	Phellopterin	002543-94-4	
15	Epoxybergamottin	206978-14-5	

Important Note: This procedure should only be applied to the quantification of furocoumarins in simple cases (essential oils), above a quantification limit (LOQ) of 10 mg/L. It is not suitable for their quantification in mixtures of essential oils, compounded fragrance oils and consumer products such as cosmetics.

- # Procedure

1.1.Materials

Analytes:

- Pure furocoumarins (minimum 95% purity).
- Possible suppliers: Chromadex (Irvine, CA), Herboreal (Edinburgh, UK).

Prepare a stock solution (1000 mg/L) of all furocoumarins in acetonitrile. Alternatively, a certified and ready-to-use stock solution can be supplied by Chromadex (Irvine, CA).

Solvents:

- Water, acetonitrile, tetrahydrofuran, methanol of HPLC grade.

Additional material:

- Alumina (e.g. Fluka 06300).

1.2.HPLC Conditions

1.2.1 Instrument:

HPLC separations have to be carried out with an HPLC system that is at least equivalent to an *Agilent* 1100 Series. Older instruments are not suitable. It should be equipped with an autosampler, a thermostatted column oven and a diode array detector (DAD).

1.2.2 Analytical HPLC method:

- The gradient separations are carried out using an *Interchim* MS Uptisphere 3 ODB (3 µm, 120A) cartridge column (150 x 2.1 mm I.D.) from *Laubscher Labs*, part number UP3ODB#15QS (Miecourt, Switzerland).
- The column is maintained at 30°C.
- Solvent A is water-acetonitrile-THF (85:10:5 v/v) and solvent B acetonitrile-methanol-THF (65:30:5 v/v). To remove the peroxides, the THF should be

freshly filtered through a plug of alumina. It is advised to weigh the solvents for the preparation of eluent A (850 g H_2O, 78.6 g ACN, 44.4 g THF) and eluent B (510.9 g ACN, 237 g MeOH, 44.4 g THF).

- The eluents should be freshly prepared prior to use, in filled, sealed bottles of maximum 1 L, and stored preferably, under an inert gas.

  .

- Gradient Profile:

Time(mins)	Solvent A	Solvent B
0-5 (isocratic)	100%	0%
5-20 (linear)	68%	32%
20-24 (isocratic)	68%	32%
24-38 (linear)	45%	55%
38-40 (linear)	10%	90%
40-50 (isocratic, rinsing)	10%	90%
50-60(equilibration)	100%	0%

- Total analysis time 60mins.
- The flow rate is 0.3 mL/min.
- The injection volume is 5 µl.
- The quantification wavelength is 310 nm. If the DAD is equipped with automatic baseline subtraction, it should be disabled.
- Calibration curves are constructed by using the calibration solutions obtained from Chromadex.

- **Suitability test**

Prior to analysis, the suitability of the system should be tested using two different test mixes

2.1. Commercial test mix

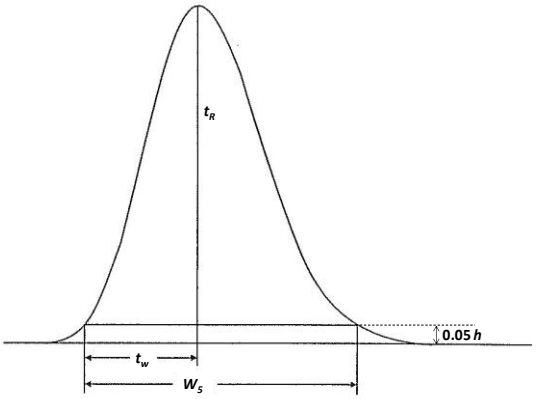
The "HPLC Gradient System Diagnostics Mix" (Supelco, reference: 48271). From the injection of this mix under the conditions described in 1.2.2, calculate:

- The number of theoretical plates, N
- The tailing factor, *t*

$$t = \frac{W_{0.5}}{2.t_W}$$

Where

- W_s = Peakwidth at 5% of the peak height
- t_W = distance between peak front and the retention time t_R at 5 % of peak height;
- $W_{0.5}$, t_W, W_5 must be expressed in the same time unit(*e.g.* minutes).



Note: In Agilent workstations, this parameter *t* is called "USP Tailing Factor"

2.2 Furocoumarin test

Inject 5 µL of the standard solution at 10 mg/L (see 3.1) under the elution conditions described in 1.2.2. Determine the tailing factor for each furocoumarin and the resolution (R_S) for each critical pair. The latter should not be less than 1.

• Quantification

3.1. Calibration

From the standard solutions in acetonitrile prepare the following calibration solutions in acetonitrile:

Volume required	Starting solution	Dilution in acetonitrile	Calibration level
250 µL	Using **1000** mg/L solution	Dilute to 10 mL	25 mg/L
100 µL	Using **1000** mg/L solution	Dilute to 10 mL	10 mg/L
10 µL	Using **1000** mg/L solution	Dilute to 10 mL	1 mg/L

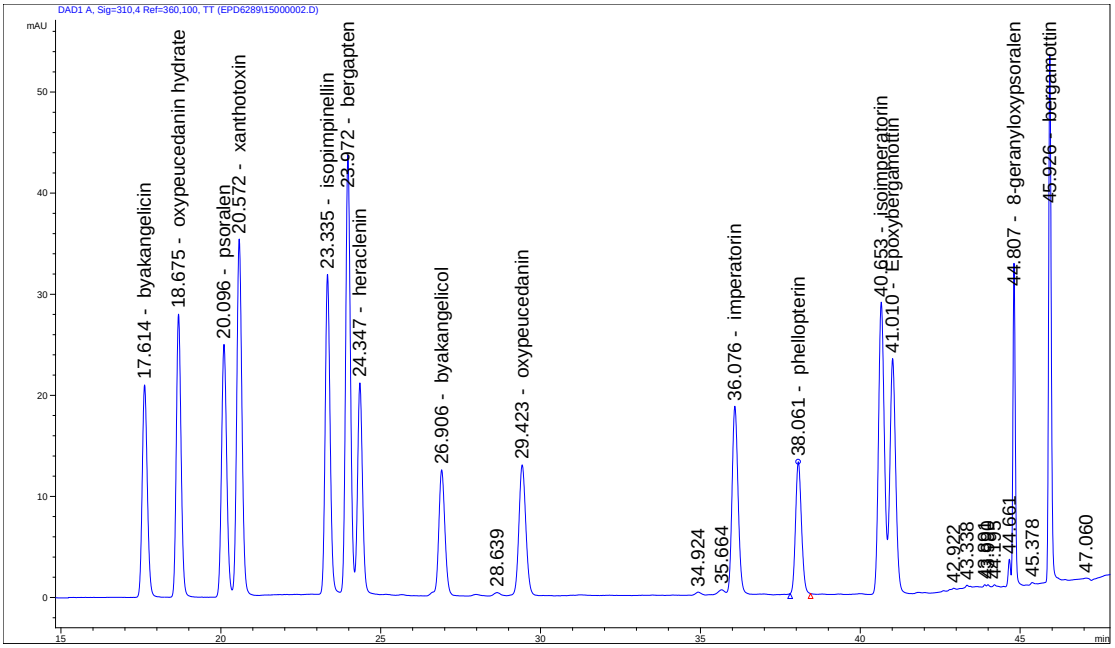
Inject the calibration solutions in a random order of concentration and inject blanks between calibration samples to prevent any carry-over.

Calibration curves are obtained by external calibration using the analytical method described in the 1.2. section, and injecting 5 µl of the calibration solutions. The equation for the calibration curve should be y = ax + b, without forcing it through zero, and weighted 1/x regarding the concentration.

Furocoumarin peak areas detected in blanks should not exceed 50% of the area of the lowest calibration solution.

UV spectra: record a spectrum (between 210 and 400 nm) of the 15 furocoumarins in the spectral library of the detector, using the standard solutions at 10 mg/L.

Figure 1. *Typical chromatogram of furocoumarins using the present conditions*



3.2. Analysis of Oils

3.2.1. Sample preparation

The samples to be quantified must be diluted 1 part into 9 parts (v/v) in acetonitrile prior to the injection.

3.2.2. Sample analysis

All these samples should be analysed using the HPLC method as described in the 1.2. section and quantified using the calibration curves.

The injection volume is 5 µl. Blanks should be injected between sample solutions to prevent any carry-over. Check the peak areas. If the concentration of one or several furocoumarin(s) exceed(s) the upper limit of the calibration range, then dilute the sample in acetonitrile down to the appropriate level and repeat the analysis.

The identity of all analytes must be checked by comparing the spectra with those of the authentic compounds recorded in the database. Quantification can only be considered to be valid if the retention time corresponds to that of the standard compound (±5%) and the similarity is at least 90% of total similarity

according to the algorithm associated with a given DAD[1]. Lower values indicate a possible co-elution.

3.3. Reporting the results

For all analytes, provide:

- The quantification of all detected furocoumarins using the calibration curves obtained with the standard solutions (external standardization).
- The match quality/similarity index of the analyte, using the reference spectrum recorded in the library under the same condition.

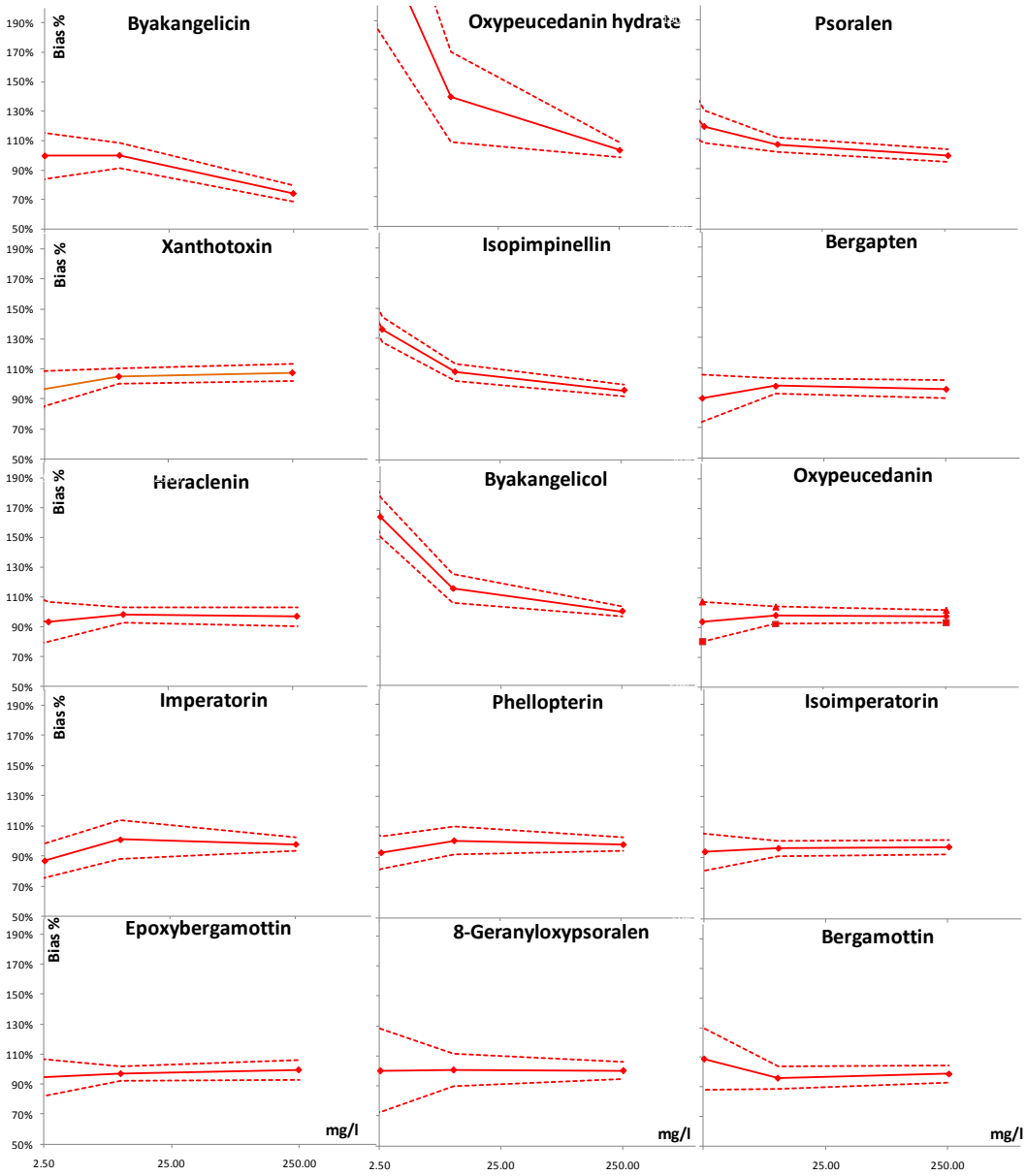
• Method performance

The validation of the present method has been fully described elsewhere [2], and only the main figures of merit are reported hereafter.

An inter-laboratory ring test has been performed to generate the accuracy profile for each of the 15 furocoumarins (Figure 2). Except oxypeucedanin hydrate, the biases and associated confidence intervals are satisfactory for concentrations above 10 mg/L.

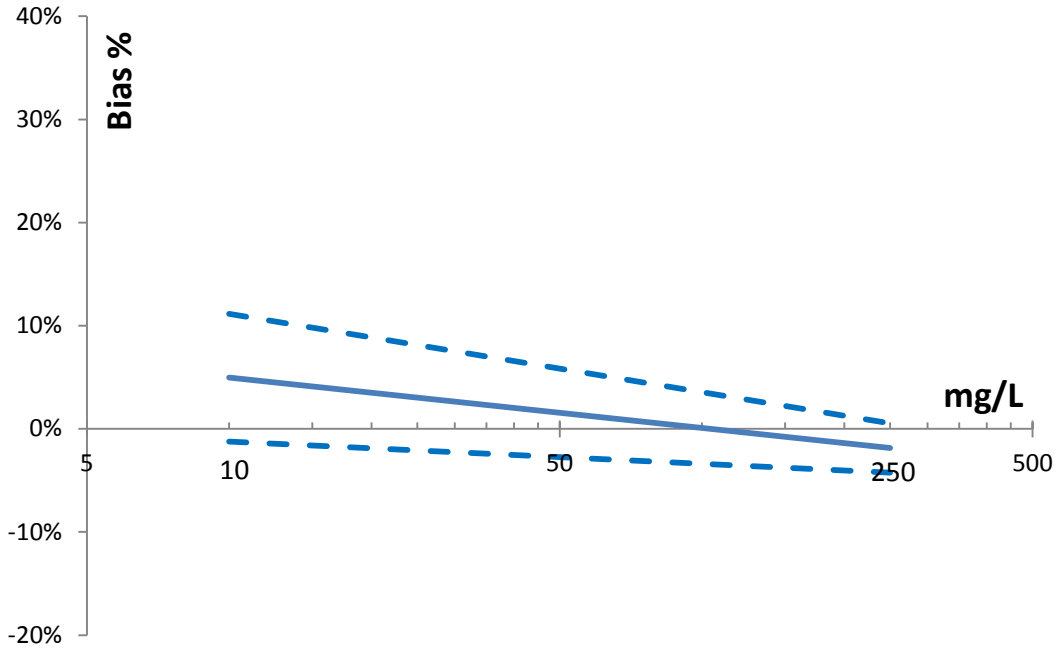
Figure 2. *Accuracy profiles: Recoveries (solid lines) and confidence intervals at the 90% level (dotted lines) (logarithmic concentration axes, 8 participants)*

[1] E.g. Agilent : match quality > 900



From the results above, the mean bias and the mean prediction profile have been determined over all analytes. Each laboratory confirmed the analyte identity thanks to its similarity to the UV spectrum recorded in the spectral library. The interval between the upper and the lower limit indicates the range in which future measurements should fall, with a probability of 90% (Figure 3).

Figure 3. *Mean prediction profiles at a confidence of 90 % when the match quality of all analytes is > 900 (15 furocoumarins, 3 laboratories, excluding one outlying value of oxypeucedanin hydrate at 10 mg/L).*



An essential oil model was spiked with all furocoumarins at various concentrations between 10 and 250 mg/L. All results are satisfactory. The small bias of oxypeucedanin hydrate and its standard deviation similar to that of other analytes suggest that the bias and variability observed in Figure 1 were not systematic.

Table 1. *Quantification of an essential oil model spiked with furocoumarins above the LOQ. Mean results over 8 participants.*

Furocoumarin	Spiking (mg/L)	Found (mg/L)	RSD (%)	Bias (%)
Byakangelicin	114.3	101.5	14.2	11
Oxypeucedanin hydrate	99.0	102.2	12.2	-3
Psoralen	45.3	44.1	11.7	3
Xanthotoxin	39.2	44.4	12.6	-13
Isopimpinellin	62.9	59.9	9.4	5
Bergapten	206.1	198.6	9.3	4
Heraclenin	23.8	25.9	25.3	-9
Byakangelicol	45.0	44.8	13.7	1
Imperatorin	151.5	148.5	8.4	2
Phellopterin	135.2	130.6	8.4	3
Isoimperatorin	68.8	65.9	10.0	4
Epoxybergamottin	71.6	70.0	10.1	2
8-Geranyloxypsoralen	29.8	31.9	13.1	-7
Bergamottin	90.4	89.9	10.0	0
Imperatorin	151.5	148.5	8.4	2

- # Annexes

In the case of poor peak shape, the column may be regenerated as follows (check that dichloromethane is compatible with the system used):

1. Reverse the flow of the column
2. Disconnect the detector(s)
3. Rinse 30 min with pure methanol as solvent B at 0.3 mL/min
4. Rinse 30 min with pure dichloromethane as solvent B at 0.3 mL/min
5. Rinse 30 min with pure methanol as solvent B at 0.3 mL/min
6. Rinse 45 min with the solvent B of the furocoumarins method

Following column regeneration repeat the system suitability test and check peak shape by injecting the standard furocoumarin solution.

Reference List

[1] E.Frerot, E.Decorzant, J. Agric. Food Chem. 52 (2004) 6879.

[2] A.Macmaster, N.Owen, S.Brussaux, H.Brévard, R.Hiserodt, H.Leijs, N.Bast, B.Weber, G.Loesing, A.Sherlock, C.Schippa, M.Vey, E.Frerot, E.Tissot, A.Chaintreau, J. Chromatog. A, 1257 (2012) 34– 40.