
**Traditional Chinese medicine —
Determination of benzopyrene in
processed natural products**

*Médecine traditionnelle chinoise — Dosage du benzopyrène dans les
produits naturels transformés*

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 249, *Traditional Chinese medicine*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

The International Agency for Research on Cancer (IARC) classified benzopyrene (C₂₀H₁₂) as a carcinogen (Group 1) in 2006.^[1] Benzopyrene is produced by the incomplete combustion of carbohydrates, proteins and fats during high-temperature cooking and processing of raw materials. To prevent hazards induced by benzopyrene, it is important to determine the benzopyrene content in processed natural products used in traditional Chinese medicine (e.g. processed *Rehmannia* root, *Cyperus* rhizome and mume fruit), which are processed by the application of high temperatures (200 °C to 600 °C).

Some national pharmacopeias have technical guidelines for the determination of benzopyrene content^[2,3]. This document describes an analytical method, including the device specifications, chemical reagents, operation procedures and formulae to determine benzopyrene content, considering each national pharmacopeia, as well as giving references to other standardized testing methods, maximum limits and risk assessment.

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Traditional Chinese medicine — Determination of benzopyrene in processed natural products

1 Scope

This document specifies the method for the determination of benzopyrene content in processed natural products.

It is applicable to processed natural products such as processed *Rehmannia* root, processed *Cyperus* rhizome, processed ginseng and processed mume fruit. It is not applicable to the analysis of minerals used in traditional Chinese medicine.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <https://www.electropedia.org/>

3.1 benzopyrene

$C_{20}H_{12}$

organic compound of pentacyclic hydrocarbons made of pyrene and phenylene group

Note 1 to entry: The chemical information of benzopyrene is described in [Annex A](#). National regulations and limitations of benzopyrene are given in [Annex B](#).

3.2 polycyclic aromatic hydrocarbon PAH

compound that contains two or more fused aromatic rings made of only carbon and hydrogen atoms

[SOURCE: ISO 11338-1:2003, 3.3]

3.3 high-performance liquid chromatography HPLC

technique in analytical chemistry used to separate, identify and quantify each component in a mixture

Note 1 to entry: High-performance liquid chromatography relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts differently with the adsorbent material, resulting in different flow rates for different components, thereby leading to the separation of components as these flow out of the column.

3.4
gas chromatography
GC

analytical technique used to separate and determine the components of complex mixtures based on partitioning between gas and stationary phases

[SOURCE: ISO 11504:2017, 3.8, modified — definition revised.]

3.5
fluorescence detector
FLD

device used to measure the parameters of fluorescence, its intensity and wavelength distribution of the emission spectrum after excitation by a specific wavelength of light

Note 1 to entry: These parameters are used to identify the presence and amounts of specific molecules in a medium. Modern fluorometers are capable of detecting fluorescent molecule concentrations of as low as one part per trillion. Fluorescence analysis should be orders of magnitude more sensitive than other techniques. Applications include monitoring in the fields of chemistry, biochemistry, medicine and environmental sciences.

3.6
mass spectrometry
MS

analytical technique that ionizes chemical species and sorts of ions based on their mass-to-charge ratios

4 Test methods

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4.1 Reagents and apparatus

4.1.1 Reagents

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4.1.1.1 Benzo[a]pyrene (BaP) standard [high-performance liquid chromatography (HPLC) grade].

4.1.1.2 3-Methylcholanthrene, HPLC grade as an internal standard.

4.1.1.3 n-Hexane, ethanol, dichloromethane and acetonitrile (HPLC grade).

4.1.1.4 Water (or equivalent) referred to as deionized water (DIW).

4.1.2 Apparatus

4.1.2.1 Rotary vacuum evaporator.

4.1.2.2 Visible nitrogen sample concentrator.

4.1.2.3 Solid phase extraction (SPE) tube vacuum manifolds.

4.1.2.4 Rotor-stator homogenizer (mechanical homogenizer).

4.1.2.5 Gas chromatography (GC) or mass spectrometry (MS).

4.2 Sample preparation

a) Add 5 g of sample powder to 100 ml water and then extract for 90 min using an ultrasonic bath with a cooling system.

- b) Add 100 ml of hexane with 1 ml of internal standard (50 µg/kg or 50 µg/l; purity ≥ 96 % for analysis grade) to a mixture by homogenization for 5 min, and extract the samples mixtures in an ultrasonic bath for 30 min.
- c) Transfer all mixtures into a separating funnel and collect the supernatants.
- d) Extract the residue twice with 50 ml of hexane as detailed in step b).
- e) Add water (50 ml; ≥ 18,2 MΩ) to the combined hexane fraction for washing and filter the hexane fraction using anhydrous sodium sulfate. Next, concentrate the hexane fraction at 45 °C using a rotary vacuum evaporator to approximately 2 ml.
- f) Wash a Florisil SPE cartridge with 10 ml of dichloromethane for activation. Clean the cartridge using 20 ml of hexane to remove dichloromethane.
- g) Transfer directly 2 ml of the extracts into the activated Florisil SPE cartridge and elute the extracts with 20 ml of hexane and dichloromethane mixture (volume ratio of 3:1).
- h) Concentrate the eluent using a visible nitrogen sample concentrator at 35 °C. Dissolve the residue in 1 ml of acetonitrile and filter the eluent through a 0,45-µm polytetrafluoroethylene (PTFE) membrane filter.
- i) Inject 10 µl of the final eluent into the fluorescence detector (FLD) for BaP analysis.

4.3 HPLC-FLD method

4.3.1 Chromatographic condition

- Column: supelcosil LC-PAH (4,6 × 250 mm, 5 µm) or equivalent.
- Column temperature: 35 °C.
- Detector: excitation at 294 nm and emission at 404 nm.
- Mobile phase: acetonitrile and water mixture (volume ratio of 8:2)
- Flow rate: 1 ml/min.

4.3.2 Identification

The retention time of the peak should be consistent with the retention time of the standard within ± 0,2 % under identical analysis conditions.

4.3.3 Construction of calibration curve

- a) Dissolve standard (BaP) and internal standard (3-methylcholanthrene) in acetonitrile (1 µg/ml each). Store this stock solution at 4 °C. Replace the stock solution every six months.
- b) Prepare standard solutions with different concentrations of 3 ng/ml, 5 ng/ml, 10 ng/ml, 20 ng/ml and 40 ng/ml with 50 ng/ml of internal standard to determine linearity.
- c) Adjust the concentration of test solution in range of the calibration curve.

4.3.4 Quantification

In the calibration curve, the y -axis represents the ratio of the peak area of BaP to that of the internal standard (A_S/A_{IS}), where A_S is the peak area of the standard in solution for the calibration curve and A_{IS} is the peak area of the internal standard in solution for the calibration curve. Calculate the content of BaP in the sample using the ratio of the peak area of BaP to that of the internal standard obtained by sample preparation (A_{SAM}/A_{SAMIS}), where A_{SAM} is the peak area of BaP in the sample extract and

A_{SAMIS} is the peak area of internal standard in the sample extract. An example of HPLC pattern about benzopyrene(s) in processed *Rehmannia* root is given in [Annex C](#).

4.4 GC-MS method

4.4.1 Chromatographic conditions

- Column: Cross-linked 5 % phenylmethylsilicone, 30 m × 0,25 mm i.d. (0,25 μm), film thickness.
- Carrier gas flow: He at 1 ml/min.
- Injection port temperature: 300 °C.
- Transfer line temperature: 280 °C.
- Oven temperature (starting step): 50 °C for 1 min.
- Oven temperature (middle step): increase 10 °C /min until 180 °C.
- Oven temperature (starting step): increase 5 °C /min until 310 °C.
- Post run: 310 °C, 3 min.

4.4.2 Assay

Use the mass spectra at 70 eV with electron impact ionization mode. Apply the electron multiplier to 300 V over the autotune values. Detect BaP and the internal standard using selected ion monitoring (SIM) mode to analyse the characteristic ions in the mass spectrum. The quantifier and qualifier ions are listed in [Table 1](#). For an example of another case, determination of benzopyrene (s) in wood creosote is described in [Annex D](#).

Table 1 — Quantifier and qualifier ions used in the selected ion monitoring of polycyclic aromatic hydrocarbons (PAHs) by GC-MS

PAH	Quantifier ion m/z	Qualifier ion m/z
Acenaphthene	154	153 152
Acenaphthylene	152	151 153
Anthracene	178	176 179
Benzo[a]anthracene	228	229 226
Benzo[b]fluoranthene	252	253 125
Benzo[k]fluoranthene	252	253 125
Benzo[g,h,i]perylene	276	138 277
Benzo[a]pyrene	252	253 125
Chrysene	228	226 229
Dibenz[a,h]anthracene	278	139 279
Fluoranthene	202	101 203
Fluorene	166	165 167
Indeno[1,2,3-cd]pyrene	276	138 227
Naphthalene	128	129 127
1-Methylnaphthalene	142	115 141
2-Methylnaphthalene	142	115 141
Phenanthrene	178	179 176
Phenanthrene-d ₁₀ (I.S.)	188	188