
**Traditional Chinese medicine —
Determination of selected *Aconitum*
alkaloids by high-performance liquid
chromatography (HPLC)**

*Médecine traditionnelle chinoise — Dosage d'alcaloïdes d'aconit
(Aconitum) sélectionnés par chromatographie liquide à haute
performance (CLHP)*

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ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

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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 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

Aconitum is a genus of about 350 species of flowering plants belonging to the family of Ranunculaceae. The therapeutic use of as many as 76 species of *Aconitum* herbs in traditional Chinese medicine have been documented for a very long time. Among the *Aconitum* herbs, the most well known are processed *Aconitum carmichaelii* lateral root (附子), *Aconitum carmichaelii* root (川乌) and *Aconitum kusnezoffii* root (草乌). Of approximately 80 000 traditional Chinese medicine formulae, around 10,2 % contain *Aconitum* herbs. It is one of the most frequently used groups of herbal medicines in traditional Chinese medicine. Typical *Aconitum* herbs used in traditional Chinese medicine are shown in [Annex C](#), Table C.1.

Aconitum herbs contain *Aconitum* alkaloids which have anti-inflammatory, analgesic and cardiotonic activities. The *Aconitum* alkaloids are a double-edged sword, however. At present, international trade in *Aconitum* products is restricted to a few nations due to the high natural toxicity of crude *Aconitum* products. Unprocessed *Aconitum* is highly toxic. Expert processing is required to reduce (but not eliminate) toxicity, and where individual national regulatory schemes do not ban the herb, it is generally restricted to a high-risk category, such as Schedule 2 in the Hong Kong Chinese Medicine regulations. Also, there are sporadic cases of *Aconitum* alkaloid poisoning due to misuse reported all over the world.

Nonetheless, the toxicity of *Aconitum* herbs can be reduced dramatically with proper processing (such as repeated boiling or steaming), prolonged decoction and dose control. However, testing standards for *Aconitum* alkaloids have not yet been harmonized on an international level, and regulatory authorities in many nations do not adequately differentiate highly toxic forms from less-toxic forms (or even non-toxic forms) of *Aconitum* herbs.

Six kinds of *Aconitum* alkaloids [aconitine (AC), mesaconitine (MA), hyaconitine (HA), benzoylaconine (BAC), benzoylmesaconine (BMA) and benzoylhypaconine (BHA)] are commonly used as chemical markers for quality control of aconite, determined by the high-performance liquid chromatography (HPLC) method^[1]. The AOAC Official Method 2008.11 also requires the determination of three *Aconitum* alkaloids, AC, MA and HA, in dietary supplements and raw botanical materials by LC/UV detection with confirmation by LC/MS/MS^[2]. Nevertheless, poisoning cases are still occasionally reported. From 1989 to 2010, 140 cases of *Aconitum* poisoning, including one fatal case, were reported in Hong Kong^[3]. Additionally, 17 cases were reported in Taiwan from 1990 to 1999, 2017 cases in China from 1989 to 2008 and 121 cases in Korea from 1995 to 2007^[4]. Multiple reasons for *Aconitum* poisoning exist and include overdoses, inadequate processing, *Aconitum* contamination in other herbs, dispensing and management errors and hidden risk factors. In the 17 cases reported in Hong Kong, yunaconitine (YAC), crassicauline A (CCA) and 8-deacetyl-yunaconitine (DYA) were detected instead of AC, MA and HA in the urine samples of the *Aconitum* poisoning patients^[3,4]. As a result, these alkaloids are considered to be hidden risk factors and should be covered in laboratory screenings for toxic compounds^[5]. Therefore, a method to simultaneously determine the levels of these nine alkaloids is needed for quality control of the herb and its products in order to ensure the safe use of these medicinal materials^[6].

This document aims to build a systematic and practical international standard for the determination of *Aconitum* alkaloids with the goal of standardizing the global market, to ensure safe and effective use in clinics and to reduce cases of *Aconitum* alkaloid poisoning.

As national implementation may differ, national standards bodies are invited to modify the limit values of selected *Aconitum* alkaloids in their national standards. Examples of national and regional values are given in [Annex D](#).

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Traditional Chinese medicine — Determination of selected *Aconitum* alkaloids by high-performance liquid chromatography (HPLC)

1 Scope

This document specifies methods for the determination of the selected *Aconitum* alkaloids, including aconitine, mesaconitine, hypaconitine, benzoyleaconine, benzoylmesaconine, benzoylhypaconine, yunaconitine, deacetyl-yunaconitine and crassicauline A.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

World Health Organization, *Quality control methods for herbal materials*, 2011

3 Terms and definitions

No terms and definitions are listed in this document.

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

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

4 Abbreviated terms

For the purposes of this document, the following abbreviated terms apply.

AC	aconitine
BAC	benzoyleaconine
BHA	benzoylhypaconine
BMA	benzoylmesaconine
CAS	chemical abstracts service
CCA	crassicauline A
CRS	chemical reference substance
DYA	8-deacetyl-yunaconitine
ESI	electrospray ionization
HA	hypaconitine

HPLC	high-performance liquid chromatography
JAC	jesaconitine
MA	mesaconitine
MRM	multiple reaction monitoring
MS	mass spectrometer
TS	test solution
UV DAD	ultraviolet diode array detector
YAC	yunaconitine

5 Principle

The test solutions (TSs) are analysed by HPLC on a reverse-phase column packed with octadecylsilane bonded silica gel, with 0,1 % formic acid as the mobile phase A, acetonitrile as the mobile phase B and by mass spectrometer (MS) or ultraviolet diode array detector (UV DAD).

Aconitum alkaloids determinable by this method are shown in [Table 1](#). Relevant structural formulae of *Aconitum* alkaloids are given in [Figure 1](#).

Table 1 — *Aconitum* alkaloids determinable by HPLC method

Name	Molecular formula	CAS no.	Molar mass g/mol
AC	C ₃₄ H ₄₇ NO ₁₁	302-27-2	645,74
MA	C ₃₃ H ₄₅ NO ₁₁	2752-64-9	631,71
HA	C ₃₃ H ₄₅ NO ₁₀	6900-87-4	615,71
BAC	C ₃₂ H ₄₅ NO ₁₀	466-24-0	603,78
BMA	C ₃₁ H ₄₃ NO ₁₀	63238-67-5	589,68
BHA	C ₃₁ H ₄₃ NO ₉	63238-66-4	573,67
YAC	C ₃₅ H ₄₉ NO ₁₁	70578-24-4	659,76
DYA	C ₃₃ H ₅₁ NO ₁₀	110011-77-3	645,79
CCA	C ₃₅ H ₄₉ NO ₁₀	79592-91-9	643,76

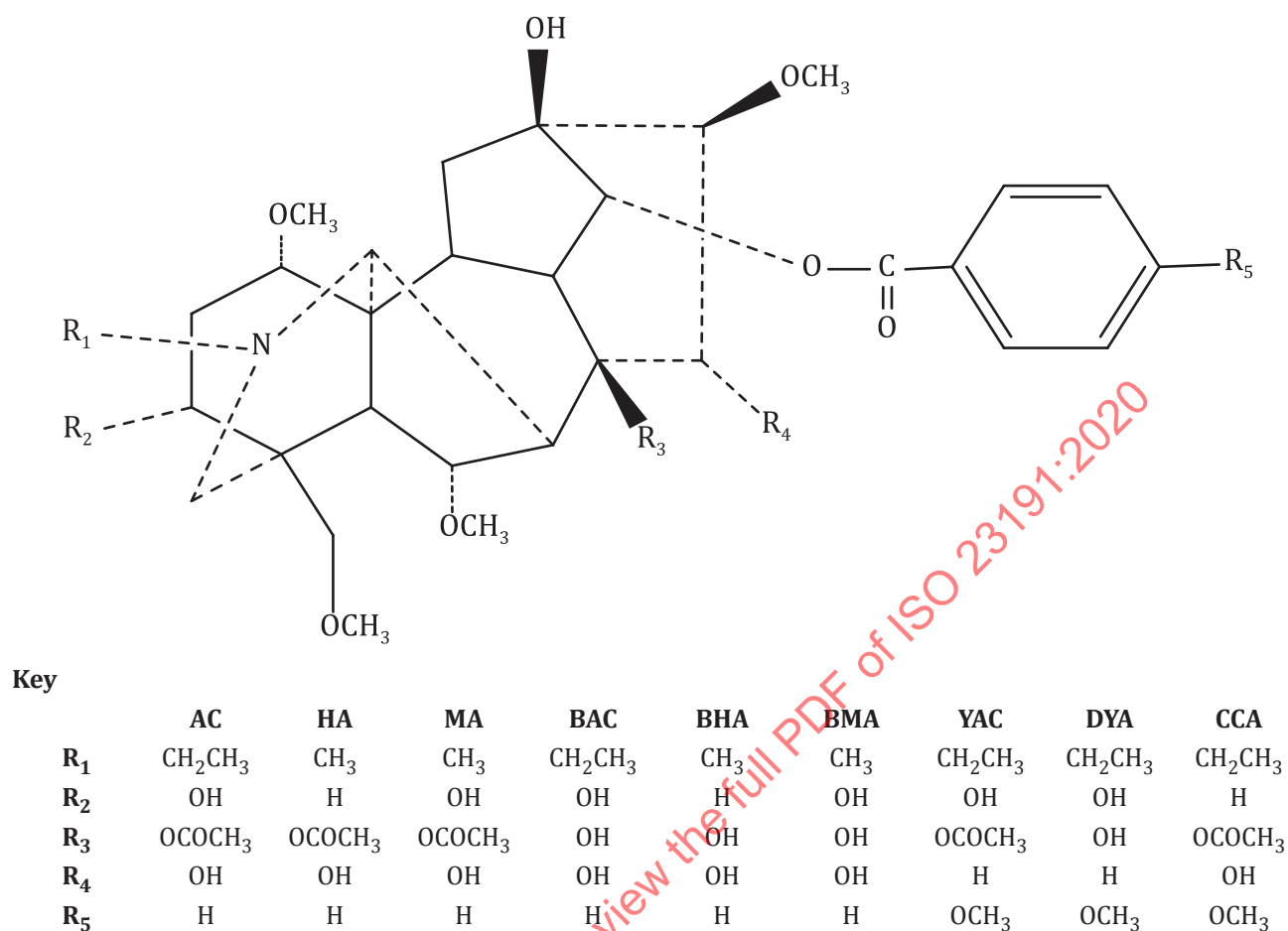


Figure 1 — Structural formulae of selected *Aconite* alkaloids

6 Reagents

Use only reagents of recognized analytical grade and water conforming with grade 3 as specified in ISO 3696, unless otherwise specified.

- **isopropanol**, C₃H₈O, AR grade;
- **dichloromethane**, CH₂Cl₂, AR grade;
- **acetonitrile**, CH₃CN, HPLC grade;
- **ethyl acetate**, C₄H₈O₂, AR grade;
- **methanol**, CH₃OH, HPLC grade;
- **purified water**, deionized;
- **ammonia TS**.

7 Apparatus

Use the usual laboratory apparatus and the following:

- **liquid chromatograph**, fitted with electrospray ionization (ESI) MS or DAD;

- **chromatographic column**, of stainless steel, 2,1 mm × 100 mm, with 1,7 µm particle size packing of octadecylsilane bonded silica gel;
- **ultrasonic bath**, power 300 W, frequency 40 kHz;
- **one-mark pipettes**, of capacity 5 ml, 25 ml and 50 ml;
- **conical flasks**, of capacity 100 ml, with glass stopper;
- **vacuum system** (e.g. Büchner flask, Vac-Elut system 1 or peristaltic pump);
- **analytical balance**, capable of weighing to the nearest 0,001 g;
- **microporous membrane**, 0,22 µm, organic phase.

8 Sampling

Sampling shall be carried out in accordance with the method described in the World Health Organization's *Quality control methods for herbal materials, General advice on sampling*. Samples not less than 250 g shall be taken from each batch randomly. Sampling of *Aconitum* genus shall be conducted as follows:

- a) from a batch of five containers or packaging units, take a sample from each;
- b) from a batch of six to 50 units, take a sample from five;
- c) from a batch of over 50 units, sample 10 %, rounding up the number of units to the nearest multiple of 10. For example, a batch of 51 units would be sampled as for 60, i.e. take samples from six packages;
- d) from each container or package selected, take three original samples from the top, middle and bottom of the container or package;
- e) the three original samples shall then be combined into a pooled sample which shall be mixed carefully;
- f) the average sample is obtained by quartering:
 - from the pooled sample, adequately mix into an even and square-shaped heap;
 - divide this diagonally into four equal parts;
 - take two diagonally opposite parts and mix carefully;
 - repeat the process as necessary until the required quantity, to within ± 10 %, is obtained;
- g) using the same quartering procedure, divide the average sample into four final samples, taking care that each portion is representative of the bulk material.

9 Test procedures

9.1 General

The HPLC-MS method applies and the HPLC-DAD method is optional. If the analytical results according to the HPLC-MS method and the HPLC-DAD method are equal, both methods can be applied. If the analytical results are different, the HPLC-MS method shall be used and is preferred for DYA, YAC and CCA because of its high sensitivity. The procedures can be modified if the accuracy and precision are validated. Other similar methods can be applied if they are validated.

9.2 HPLC-MS procedure

9.2.1 Reference solution

Weigh accurately mesaconine chemical reference substance (CRS), hyaconitine CRS, aconitine CRS, benzoylmesaconine CRS, benzoylaconitine CRS, benzoylhyaconitine CRS, yunaconitine CRS, deacetyl-yunaconitine CRS and crassicauline A CRS and dissolve in methanol to produce a mixture of these compounds at a concentration appropriate for the determination, such as 6,56 µg per ml, 5,46 µg per ml, 7,89 µg per ml, 5,65 µg per ml, 16,5 µg per ml, 5,91 µg per ml, 4,95 µg per ml, 5,08 µg per ml and 2,53 µg per ml for the 9 CRSs, respectively.

Personal safety precautions, such as wearing a mask and gloves, should be taken when preparing the reference solution.

9.2.2 Test solution

- Crush the test samples.
- Weigh accurately 2 g of sample powder (through a 50-mesh sieve) into a conical flask with stopper.
- Accurately add 3 ml of ammonia TS and 50 ml of a mixture of isopropanol and ethyl acetate (1:1) and weigh.
- Ultrasonicate (power 300 W, frequency 40 kHz, water temperature below 30 °C) for 30 min, cool and weigh again. Replenish the loss of solvent with the mixture of isopropanol and ethyl acetate (1:1) and mix well.
- Evaporate accurately 25 ml of the successive filtrate to dryness under reduced pressure below 40 °C.
- Dissolve the residue in exactly 3 ml of a mixture of isopropanol and dichloromethane (1:1) and mix well.
- Dilute with methanol in a 1:10 ratio and mix well.
- Filter with a 0,22 µm microporous membrane filter and use the filtrate as the test solution.

Personal safety precautions, such as wearing a mask and gloves, should be taken when preparing the test solution.

9.2.3 Chromatographic conditions

Typical chromatographic conditions are shown in [A.1](#).

9.2.4 HPLC-MS conditions

Typical HPLC-MS conditions and ions monitored are shown in [A.1](#).

9.2.5 Determination

Inject accurately 2 µl each of the reference solution and the test solution into the column and calculate the content of each alkaloid in the test sample solution against the reference solution. The chromatograms and methodological contents are shown in [B.1](#).

9.3 HPLC-DAD procedure

9.3.1 Reference solution

- Weigh accurately MA CRS, HA CRS and AC CRS, then dissolve in a mixture of isopropanol and dichloromethane (1:1) to produce a mixture containing 5 µg of each per ml.

- b) Dissolve BMA CRS, BAC CRS and BHA CRS in a mixture of isopropanol and dichloromethane (1:1) to produce a mixture containing 10 µg of each per ml.
- c) Dissolve YAC CRS, DYA CRS and CCA CRS in a mixture of isopropanol and dichloromethane (1:1) to produce a mixture with concentrations of 4,95 (YAC), 5,08 (DYA) and 2,53 (CCA) µg/ml, respectively.

Personal safety precautions, such as wearing a mask and gloves, should be taken when preparing the reference solution.

9.3.2 Test solution

- a) Weigh accurately 2 g of the powder (through a 50-mesh sieve) into a conical flask with stopper.
- b) Accurately add 3 ml of ammonia TS and 50 ml of a mixture of isopropanol and ethyl acetate (1:1) and weigh.
- c) Ultrasonicate (power 300 W, frequency 40 kHz, water temperature below 30 °C) for 30 min, cool and weigh again. Replenish the loss of solvent with the mixture of isopropanol and ethyl acetate (1:1) and mix well.
- d) Evaporate accurately 25 ml of the successive filtrate to dryness under reduced pressure below 40 °C.
- e) Dissolve the residue in exactly 3 ml of a mixture of isopropanol and dichloromethane (1:1) and mix well.
- f) Filter and use the filtrate as the test solution.

Personal safety precautions, such as wearing a mask and gloves, should be taken when preparing the test solution.

9.3.3 Chromatographic conditions

Typical HPLC-DAD conditions are shown in [A.2](#).

9.3.4 Determination

Inject accurately 5 µl each of the reference solution and the test solution into the column and calculate the content of each alkaloid in the test sample solution against the reference solution. The chromatograms are shown in [B.2](#).

10 Test report

For each test method, the test report shall specify the following:

- a) all information necessary for the complete identification of the sample;
- b) the sampling method used;
- c) the test method used;
- d) a reference to this document, i.e. ISO 23191:2020;
- d) the test result(s) obtained;
- e) all operating details not specified in this document, or regarded as optional, together with details of any incidents which may have influenced the test result(s);
- f) any unusual features (anomalies) observed during the test;
- g) the date of the test.

Annex A (informative)

Typical HPLC conditions

A.1 Typical HPLC-MS conditions and ions monitored

The parameters can vary depending on the particular condition of different instruments, and the following parameters are for reference only. Typical chromatographic conditions are shown in [Table A.1](#). Typical MS conditions are shown in [Table A.2](#). Ions monitored are shown in [Table A.3](#).

Table A.1 — Typical chromatographic conditions

Stationary phase	Octadecylsilane bonded silica gel (C ₁₈ column 1,7 µm, 2,1 mm × 100 mm)		
Mobile phase A	0,1 % formic acid		
Mobile phase B	Acetonitrile		
Gradient	Time (min)	Mobile phase A (per cent volume fraction)	Mobile phase B (per cent volume fraction)
	0–4	80→75	20→25
	4–10	75	25
	10,01–12	10	90
	12,01–15	80	20
Flow rate	0,35 ml/min		
Injection volume	2 µl		
Temperature column oven	Ambient temperature		
The number of theoretical plates of the column	≥ 3 000 ^a .		
^a Calculated with reference to the peak of benzoylmesaconine.			

Table A.2 — Typical MS conditions

Ionization:	ESI positive
Mode:	Multiple reaction monitoring (MRM)
Drying gas:	N ₂
Flow rate:	11,0 l/min
Drying gas temperature:	300 °C
Nebulizer:	15 psig
Capillary voltage:	4 kV

Table A.3 — Ions monitored

Component	Parent ion m/z	Daughter ion m/z	Collision energy eV
AC	646,3	586,3	33
MA	632,3	105,1	50
HA	616,3	556,3	29
BAC	604,3	105,1	50

Table A.3 (continued)

Component	Parent ion m/z	Daughter ion m/z	Collision energy eV
BMA	509,3	105,1	49
BHA	574,3	105,1	50
YAC	660,3	135,0	64
DYA	663,4	495,3	52
CCA	644,4	135,1	68

A.2 Typical HPLC-DAD chromatographic conditions

The parameters can vary depending on the particular condition of different instruments, and the following parameters are for reference only. Typical HPLC-DAD chromatographic conditions are shown in [Table A.4](#). The detector is a spectrophotometer set at 235 nm.

Table A.4 — Typical HPLC-DAD chromatographic conditions

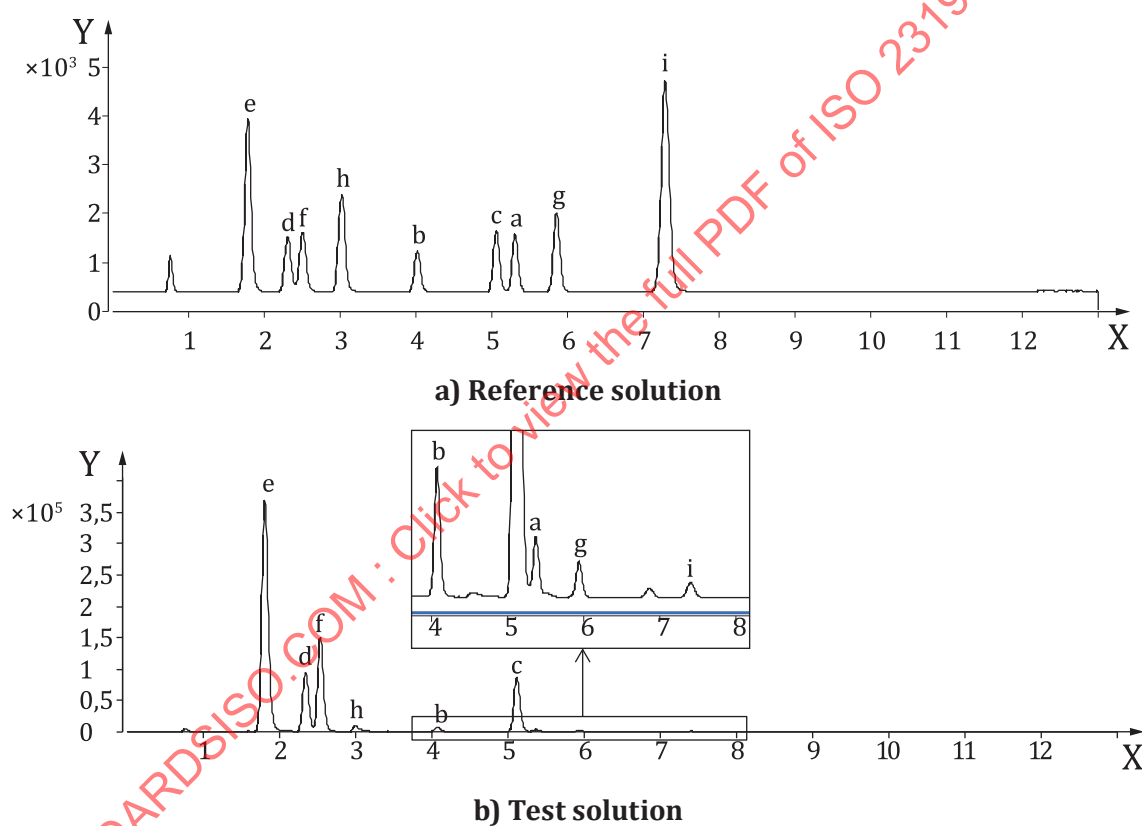
Stationary phase	Octadecylsilane bonded silica gel (C ₁₈ column 1,7 µm, 2,1 mm × 100 mm)		
Mobile phase A	0,1 % formic acid		
Mobile phase B	Acetonitrile		
Gradient	Time (min)	Mobile phase A (per cent volume fraction)	Mobile phase B (per cent volume fraction)
	0–10	84→77	16→23
	10–15	77	23
	15–16	77→10	23→90
	20	10	90
Flow rate	0,35 ml/min		
Injection volume	5 µl		
Temperature column oven	Ambient temperature		
The number of theoretical plates of the column	≥ 3 000 ^a		
^a Calculated with reference to the peak of benzoylmesaconine.			

Annex B (informative)

Typical chromatogram of the selected *Aconitum* alkaloids

B.1 HPLC-MS method

Representative MRM chromatograms of the reference solution (a) and test solution (b) are shown in [Figure B.1](#). Related linearity, range and limits of determination and quantification are shown in [Table B.1](#).



Key

X	time, min	e	BHA
Y	intensity, cps	f	BMA
a	AC	g	YAC
b	HA	h	DYA
c	MA	i	CCA
d	BAC		

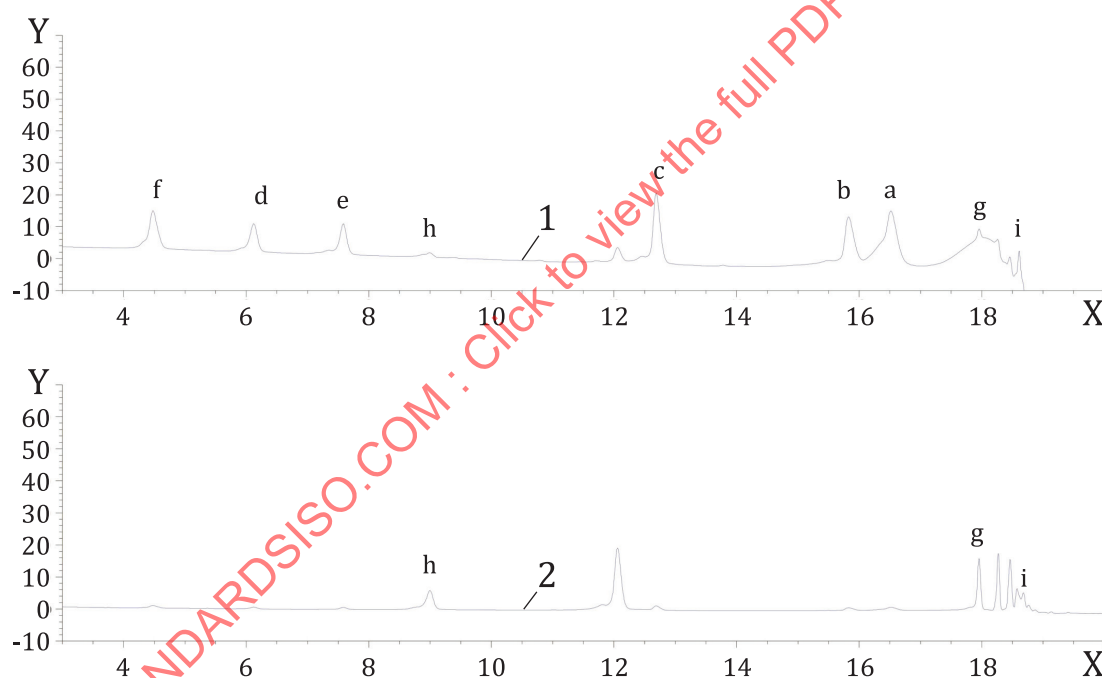
Figure B.1 — Representative MRM chromatograms of the reference solution (a) and test solution (b) of processed *Aconitum carmichaelii* lateral root

Table B.1 — Linearity, range and limits of determination and quantification

Analyte	Linearity	R	Range ng/ml	LOD ng/ml	LOQ ng/ml
AC	$Y = 20\,447X + 6\,476$	0,999 7	0,656 to 6 560	0,131	0,656
MA	$Y = 19\,008X + 4\,958$	0,999 7	0,546 to 5 460	0,109	0,546
HA	$Y = 18\,548X + 8\,882$	0,999 5	0,789 to 7 890	0,158	0,789
BAC	$Y = 22\,899X + 9\,210$	0,999 4	0,565 to 5 650	0,113	0,565
BMA	$Y = 19\,125X + 31\,020$	0,999 0	1,65 to 16 500	0,330	1,650
BHA	$Y = 24\,881X + 10\,680$	0,999 3	0,591 to 5 910	0,118	0,591
YAC	$Y = 385\,584X + 5\,933$	0,999 8	0,495 to 4 950	0,099	0,495
DYA	$Y = 42\,795X + 8\,856$	0,999 8	0,508 to 5 080	0,102	0,508
CCA	$Y = 45\,812X - 1\,659$	1,000 0	0,253 to 2 530	0,051	0,253

B.2 HPLC-DAD method

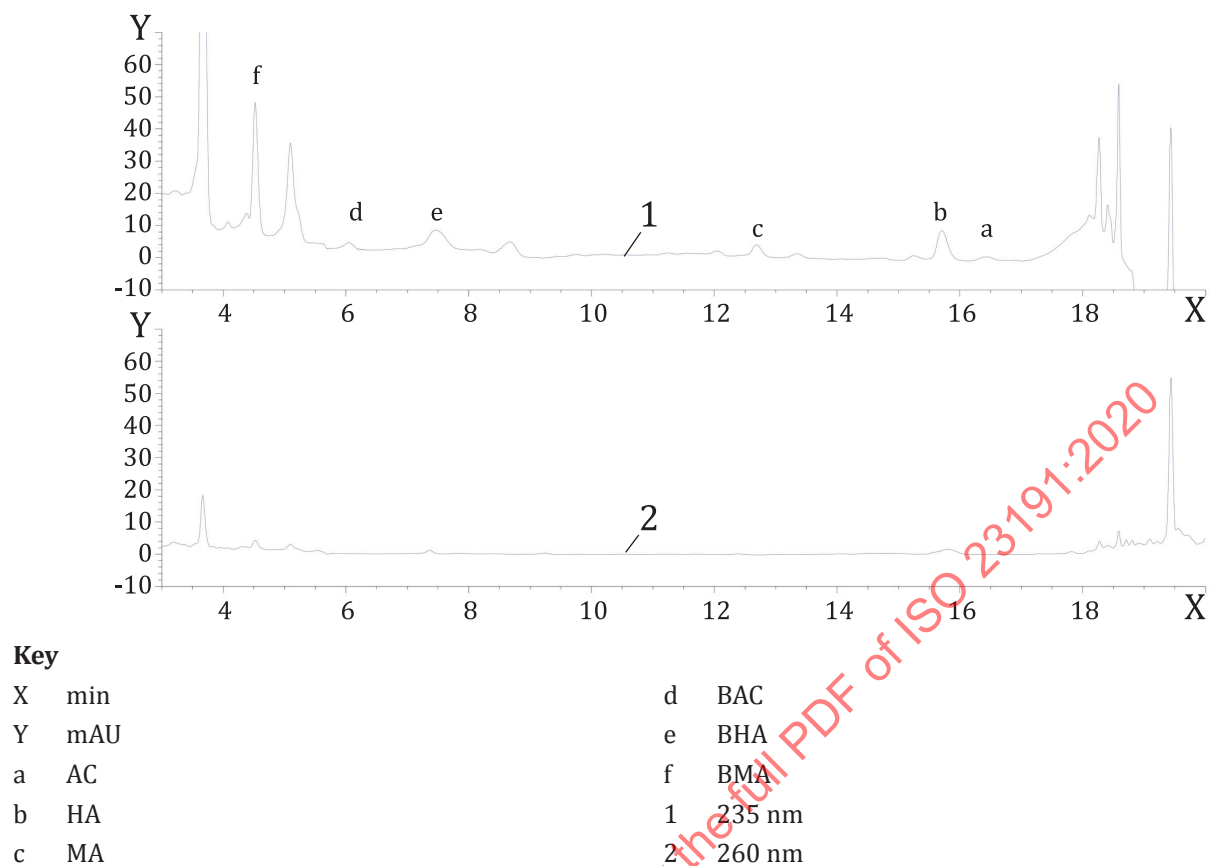
Typical HPLC-DAD chromatograms of reference solution are shown in [Figure B.2](#). Typical HPLC-DAD chromatograms of test solution are shown in [Figure B.3](#).



Key

X	min	f	BMA
Y	mAU	g	YAC
a	AC	h	DYA
b	HA	i	CCA
c	MA	1	235 nm
d	BAC	2	260 nm
e	BHA		

Figure B.2 — Typical HPLC-DAD chromatograms of standard compounds



NOTE Chromatogram peaks of DYA, YAC and CCA are not detected, because their content is below the lower limit of quantification and detection. HPLC-MS method is preferred for DYA, YAC and CCA because of its high sensitivity.

Figure B.3 — Typical HPLC-DAD chromatograms of test solution