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Research Article

HPLC Method for Determination and Optimization of 2,4-DNPH Pre-Column Derivatization Effect of Reaction Conditions for Muscone in Natural Musk

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ABSTRACT

To establish a method for the determination of muscone in natural musk by pre-column derivatization HPLC with 2, 4-Dinitrophenyl hydrazine (DNPH) and the reaction conditions were optimized. The influencing factors of derivatization reaction were investigated by single factor, and the main influencing factors were optimized by orthogonal test. Under the optimum reaction conditions, muscone-DNPH derivatives were obtained and detected by 365 nm following the Alltima C18 column (5 μm, 150 mm × 4.6 mm) separation. Single factor investigation revealed: the suitable reaction medium and catalyst were ethanol and hydrochloric acid, respectively; the molar ratio of muscone to DNPH should not be less than 300 times, the appropriate reaction time was 20 min or more; the effect of reaction temperature on derivatization reaction was obvious and needs to be optimized. Furthermore, the orthogonal test of acid concentration, reaction time and temperature showed that the effect of temperature on reaction was significant ($p<0.05$). The influence of the other two factors were not obvious ($p>0.05$); The optimum reaction conditions were: reaction temperature was 65°C, hydrochloric acid be added to derivatization solution was (1.25:98.75) (mL/mL) and the reaction time was 30 min. Under this conditions, there was good linearity in range of 0.04 μg•mL-1-30.00 μg•mL-1 for muscone ($r=0.999\ 9$), the average spiked recoveries were 98.37%-100.32% and their RSD were 0.57%-1.32%. All the RSD of repeatability of sample and standard treatment were less than 4.12%; the limits of detection (S/N=3) and quantitation (S/N=15) were 0.005 μg•mL-1 and 0.04 μg•mL-1 respectively. The established method for detecting is accurate with good reproducibility and can be used for determination of muscone in natural musk.

Keywords: DNPH; Pre-column derivatization; Muscone; Natural musk; HPLC; Optimization

INTRODUCTION

Due to the lack of a conjugated structure in the muscone molecule, the ultraviolet region absorbs between 190 and 200 nm, and most organic solvents also have strong background absorption in this region. The HPLC-ultraviolet method cannot detect the musk directly, due to its ketone content. Therefore, the content of muscone in natural musk is currently determined by gas chromatography (GC) [1-5] or gas chromatography-mass spectrometry (GC-MS) [6,7].

2.4-Dinitrophenylhydrazine (DNPH) is a commonly used derivatization reagent for aldehydes or ketones. Its derivatized product, phenylhydrazine, has a good UV response at around 360 nm, which means it is low in the environment, food and pharmaceutical fields. It is widely used in the detection of aldehydes [8-13]. Muscone is a natural 3-methylcyclopentadecanone which can be combined with DNPH. The quantitative conversion relationship between the product muscone benzoquinone and the reaction substrate muscone can be used for HPLC quantitative determination of muscone, as shown in Figure 1 below [14-16].

DNPH derivatization of aldehydes and ketones is a reversible nucleophilic reaction process. In order to completely convert the reaction substrate to phenylhydrazine, the reaction conditions need to be optimized [17].

The quantitative determination of DNPH derivatization of

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muscone in musk has been reported in the 1990s [14], but the optimization of the derivatization reaction conditions has not been reported until now. In this paper, the DNPH derivatization reaction of muscone was optimized by single factor and orthogonal test from the aspects of the type of medium, acid and its concentration, DNPH dosage, reaction temperature and reaction time. The results were satisfactory.

MATERIALS AND METHODS

Instrument

LC-10 A high performance liquid chromatograph with SCL-10 A system controller, DGU-12 A online degassing, LC-10 AT dual pump, CTO-10 A column oven, SIL-10 AXL autosampler and SPD-10 A visible-UV detector (Shimatsu co., Ltd.); DK-98-IIA electrothermal constant temperature water bath (Tianjin Test Instrument Co., Ltd.); AB265-S precision electronic balance (METTLER TOLEDO, 0.01 mg); VORTEX-6 vortex mixer Kylin-Bell; KQ-600E ultrasonic cleaner (Kunshan Ultrasonic Instrument Co., Ltd.); SORVALL LEGEND MICRO 17 high-speed centrifuge (Thermo Scientific). Alltima C18 5 μm, 4.6 × 150 mm high performance liquid chromatography column (Alltech).

Materials

Muscone standard (batch number: 541-91-3, GC ≥ 98%, Henan Standard Biotechnology Co., Ltd.); 2.4-dinitrophenylhydrazine (DN-A; Aladdin Industrial Corporation); acetonitrile (HPLC Grade, Fisher); absolute ethanol (AR, Tianjin Kemao Chemical Reagent Co., Ltd.); ethyl acetate (AR, Tianjin Beilian Fine Chemicals Development Co., Ltd.); concentrated sulfuric acid (AR, Tianjin Xiangyu Chemicals) Trade Co., Ltd.); glacial acetic acid (AR, Tianjin Beilian Fine Chemicals Development Co., Ltd.); concentrated hydrochloric acid (AR, Tianjin Xiangyu Chemical Industry and Trade Co., Ltd.); phosphorus pentoxide (AR, Tianjin Fuchen Chemical Reagent) Ltd.); Water is HUMAN UP-900 ultrapure water.

Mongolian musk (the contents of the wild scorpion's sacs, provided by the Mongolian Institute of Traditional Medicine and Technology, a total of two batches, the moisture content is 52 to 58% by Chinese Pharmacopoeia method. batch number: 201801, 201802). Chinese musk (the content of the sac of the domestic forest carp, originating in Sichuan Province, produced by Beijing Sanhe Pharmaceutical Co., Ltd., batch number: 75541201).

Chromatographic conditions

Column: Alltima C18 5 μm; 4.6 × 150 mm (Alltech); mobile phase: acetonitrile-water (90:10); flow rate: 1 mL/min; column temperature: 30°C; detection wavelength: 365 nm; injection volume: 20 μL. Under the above chromatographic conditions. The theoretical number of plates of muscone was 4,000 or more, and the degree of separation was greater than 4.

Muscone standard solution: Accurately weigh 10.00 mg of muscone reference substance, put it in a 10.0 mL volumetric flask, add a small amount of absolute ethanol to dissolve, continue to add absolute ethanol to the mark, shake well, that is, the final concentration is 1.00 Mg • mL^{-1}. If necessary, dilute with absolute ethanol to a different concentration and use.

Derivatization reagents: Weigh accurately 200 mg of 2-dinitrophenylhydrazine, placed in a 100 mL brown volumetric flask, add 80 mL of various solvents (pre-mixed) containing different types and concentrations of acid, sonicate, continue to add similar reagents to Scale, shake well, place at 8°C for 24 hours in the dark, filter with 0.45 μm microporous membrane for a final concentration of about 2 mg • mL^{-1}.

The test solution: Accurately weighed in a phosphorus pentoxide desiccator and dried under reduced pressure to a constant weight of musk (sludged and passed through a 40 mesh standard sieve) of about 10 mg, placed in a 5 mL volumetric flask, and added with 4 mL of 0.2% DNPH. Anhydrous ethanol solution (containing 1.25% hydrochloric acid, V/V), reacted in a constant temperature water bath at 65°C for 30 min, taken out, cooled at room temperature, added DNPH solution to the mark, shake well, take appropriate amount from it, centrifuge at 13000 r/min for 10 min. Take the supernatant and get it.

RESULTS

Optimization of derivatization reaction conditions

The reaction medium is taken as DNPH 200 mg, and the derivatization reagent solution (containing hydrochloric acid) is prepared according to the preparation of methanol, acetonitrile, absolute ethanol and absolute ethanol-ethyl acetate mixture (1:1) under "2.2.2". 1.25%, V/V). Take another 20 μL of 0.10 mg • mL^{-1} muscone reference solution, prepared according to the methanol, acetonitrile, absolute ethanol and absolute ethanol-ethyl acetate mixture, derivatize according to "2.2.3", and chromatographically analyze the reaction product, as shown in Figure 2. As a result, the peak area of muscone in acetonitrile solution was too small, indicating that the reaction was not thorough; the peak area of muscone in ethanol-ethyl acetate mixed solution was slightly lower than that of methanol and ethanol; although the peak surface of muscone in methanol and ethanol was basically the same, but the solvent peak of the methanol solution is significantly more complex than the ethanol solution, and the baseline fluctuation is large. Therefore, ethanol is preferred as the reaction medium.

Type and amount of acid

Take DNPH 200 mg, a total of 21 parts, divided into three groups (7 parts each), add different volume equivalents of glacial acetic acid, concentrated hydrochloric acid or concentrated sulfuric acid ethanol according to "2.2.2" Solution, prepare derivatization

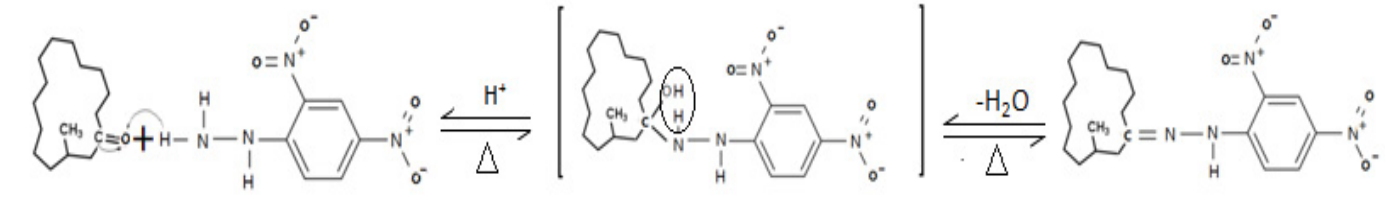
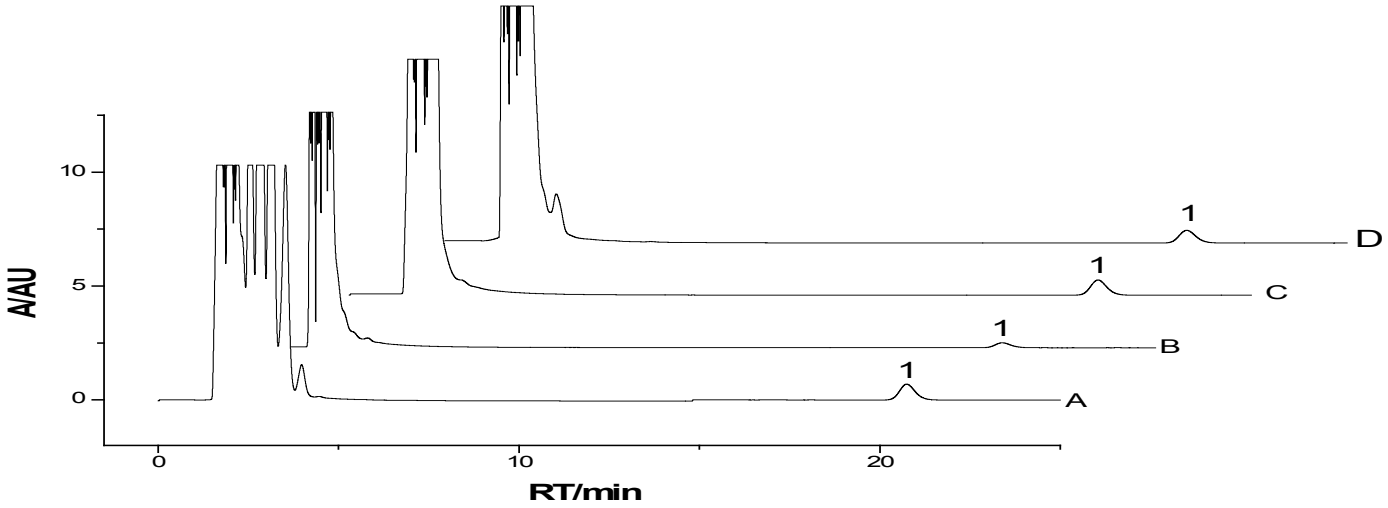


Figure 1: *Schematic diagram of muscone derivative reaction.*



Keys: A: In methanol; B: In acetonitrile; C: In ethanol; D: In ethanol-ethyl acetate; 1-Muscone.

Figure 2: HPLC chromatograms.

reagent; another 20.0 μL of muscone reference solution with a concentration of 0.10 mg•mL^{-1}, 21 parts in total, divided into three groups (7 parts each) respectively, add the above corresponding derivatization reagents. The derivatization was carried out under "2.2.3", and the reaction product was subjected to chromatographic analysis to determine the peak area of the muscone as the vertical axis, and the corresponding acid equivalent volume (per 100 mL) was plotted on the horizontal axis, as shown in Figure 3. As a result, it is difficult to completely carry out the reaction of muscone in the medium containing acetic acid, and it had a tendency to increase with the increase of the equivalent volume of the acid; the reaction in the medium containing hydrochloric acid and sulfuric acid is relatively complete, but with the increase of the volume of acid equivalent. The reaction products have a decreasing trend; since sulfuric acid has strong corrosiveness, it is suitable to select hydrochloric acid, and it is preferable to add 1-2 mL per 100 mL of ethanol.

DNPH dosage

Take the concentration of 0.10 mg•mL^{-1} muscone reference solution 20.0 μL for 5 parts, put in a 5 mL volumetric flask, add different dilutions of DNPH solution (hydrochloric acid concentration is 1.25%) 4.00 mL, the molar ratio of muscone to 2,4-dinitrophenylhydrazine is 1.2, 12, 120, 300, 600, 1200 times, respectively, and derivatization is carried out according to "2.2.3", and the reaction product is subjected to chromatographic analysis, the peak area of the muscone was measured as the vertical axis, and the corresponding molar ratio was plotted on the horizontal axis, as shown in Figure 4. As a result, when the molar ratio of muscone to DNPH is more than 300, the derivatization of muscone is relatively sufficient, and there is no correlation between the final product concentration and the amount of DNPH; if it is lower than this value, the amount of derivatized product is between DNPH andmuscone. There is a proportional increase in the trend.

Reaction time

Take a concentration of 0.10 mg•mL^{-1} muscone reference solution 20.0 μL for 5 parts, put it in a 5 mL volumetric flask, derivatize according to "2.2.3", react at 20, 40 60, 80, and 100 min respectively. The peak area of the muscone was measured as the vertical axis, and the reaction time was plotted on the horizontal axis, as shown in Figure 5. As a result, the reaction time was between 20 and 100 min, and the effect on the formation of muscone-derived compounds was not obvious. As long as the reaction time was more than 20 min, the derivatization reaction could reach equilibrium.

The reaction temperature

Taken as a concentration of 0.10 mg•mL^{-1} muscone reference solution 20.0 μL for 7 parts, placed in a 5 mL volumetric flask, derivatized according to "2.2.3", respectively at 20, 30 the reaction was carried out in a constant temperature water bath at 40, 50, 60, 70 and 75°C for 30 min. The peak area of the muscone was measured as the vertical axis, and the reaction temperature was plotted on the horizontal axis, as shown in Figure 6. As a result, at lower temperatures, the derivatization of muscone was incomplete; with the increase of reaction temperature, there was a significant strengthening trend between 40 and 60°C; and after 70°C, there was a certain degree of weakening.

Orthogonal test

According to the above single-factor gradient test, it is easy to see that the amount of hydrochloric acid is between 1 - 2 mL and the reaction temperature is between 60 - 70°C. Although the reaction time is not significant, it might be related to the reaction temperature. There was an interaction, so it is also considered as an indicator of evaluation, see Table 1.

The specific operation is as follows: precision absorption of 1.00 mg•mL^{-1} muscone reference solution 20.0 μL, a total 9 parts, respectively placed in a 5.0 mL volumetric flask, according to the above preset scheme, derivatize according to "2.2.3". The variance analysis was performed with the measured peak area value as an indicator, as shown in Tables 2 and 3. As a result, among the three factors, the effect of reaction temperature on the derivatization reaction was statistically different ($p<0.05$); the amount of hydrochloric acid and the reaction time were not statistically different in the set region ($p>0.05$); The reaction condition is A3B1C2, that is, when the amount of hydrochloric acid is 1.25 mL per 100 mL of derivatization reagent, the reaction temperature is 65°C and the reaction time is 30 min.

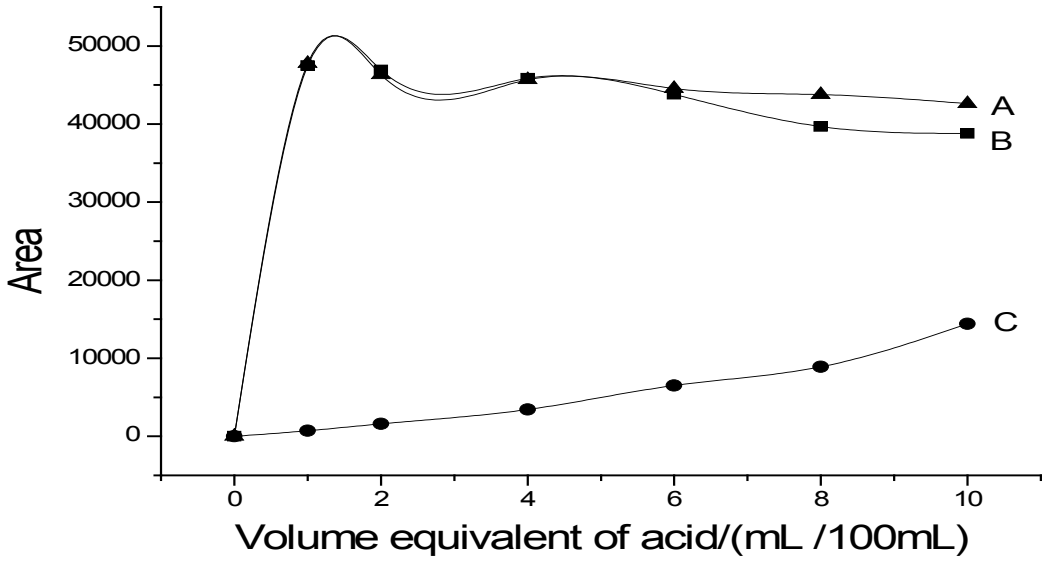


Figure 3: Effect of acid volume in DNPH ethanol solution. Keys: A: In sulfuric acid; B: In hydrochloric acid; C: In glacial acetic acid.

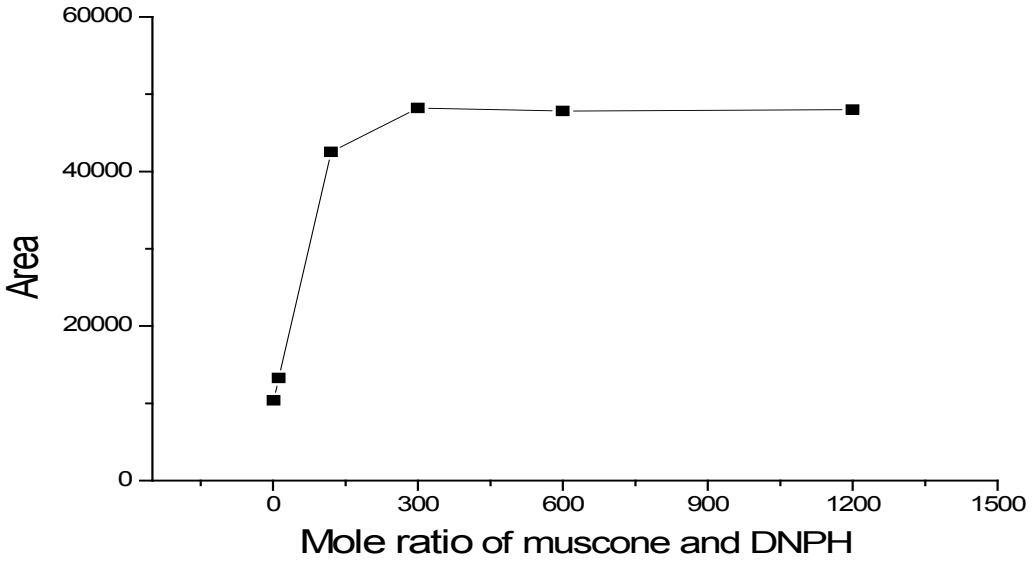


Figure 4: Effect on mole ratio of muscone and DNPH.

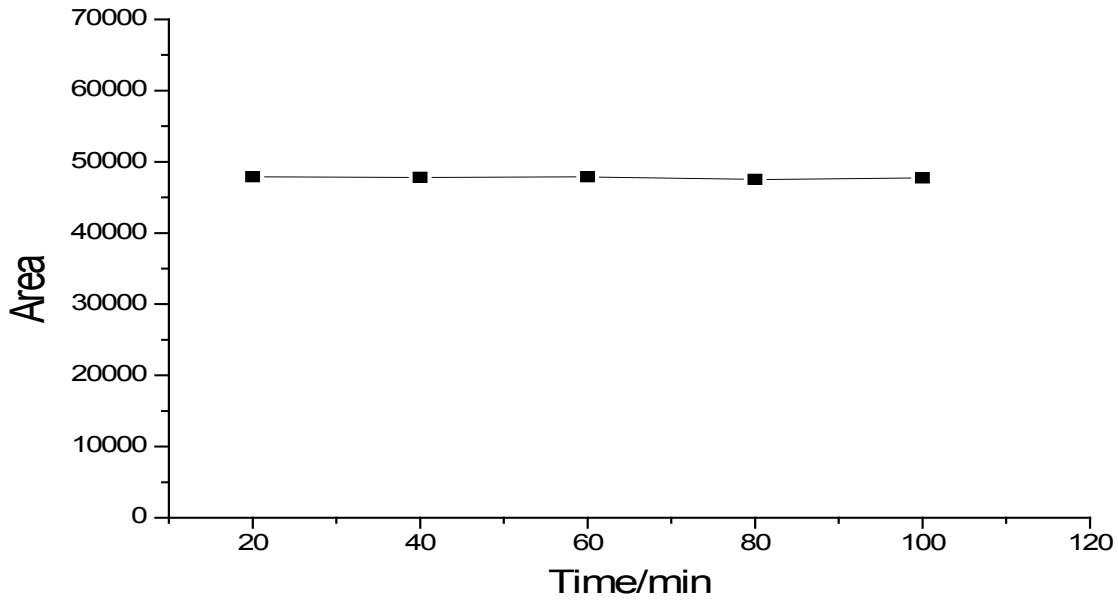


Figure 5: Effect of reaction time.

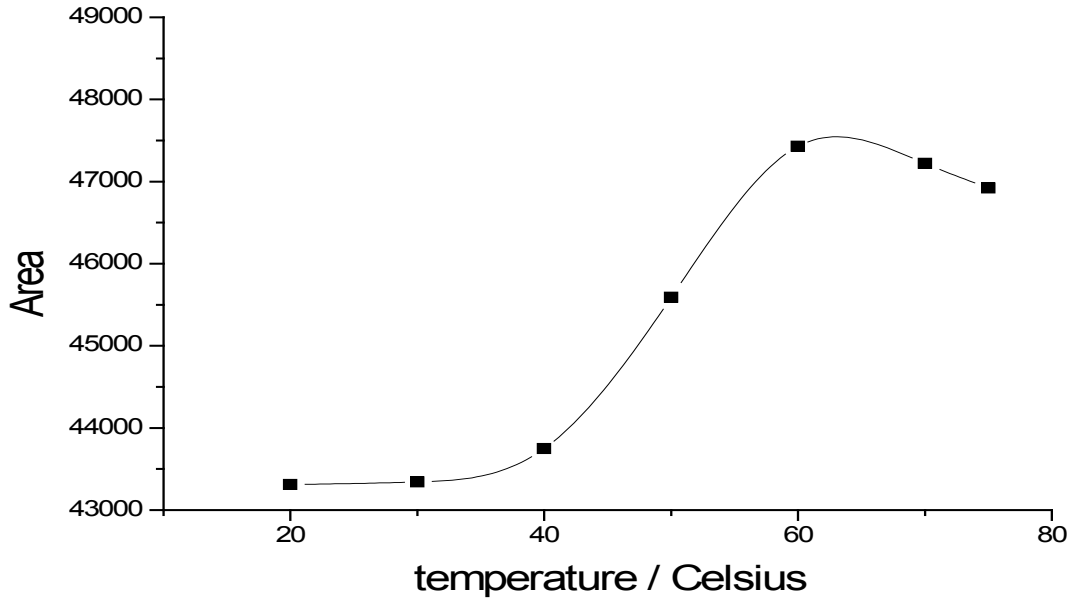


Figure 6: Effect of reaction temperature.

Table 1: $L_9(3)^4$ Orthogonal test factor and level.

Level	Factor		
	A Acid volume（mL/100 mL）	B Temperature（°C）	C Time（min）
1	0.75	65	15
2	1.00	70	30
3	1.25	75	45

Table 2: Orthogonal design and experimental results.

NO.	A	B	C	D（Error）	Measured area
1	1	1	1	1	501124
2	1	2	2	2	497938
3	1	3	3	3	471208
4	2	1	2	3	503937
5	2	2	3	1	481844
6	2	3	1	2	483249
7	3	1	3	2	507582
8	3	2	1	3	503345
9	3	3	2	1	494143
K1	1470270	1512643	1487718	1477111	G=4444370 CT=G^2/9=2194713855211
K2	1469030	1483127	1496018	1488769	
K3	1505070	1448600	1460634	1478490	
S	279051022	684979315	228273390	27052050	

Table 3: Results for Anova analysis.

Variance source	SSD	df	Mean SD	F value	P
A	279051022	2	139525511	10.32	>0.05
B	684979315	2	342489657	26.32*	<0.05
C	288273390	2	114136695	8.44	>0.05
D (Error)	27052050	2	13526025	1.00	
SUM	1219355777				

Note: F1-0.05(2,2)=19;* indicates statistical difference.

Verification test

Precision extraction concentration of 1.00 mg•mL^{-1} muscone reference solution 20.0 μL a total of 5 parts, respectively placed in a 5.0 mL volumetric flask, derivatized according to "2.2.3", as well as according to the "2.1" The chromatographic conditions were measured and the peak area values were determined and compared with the highest observed values in the orthogonal table. As a result, the measured value was 100.20% of the highest observed value, and the RSD was 1.60%, indicating that the optimal derivatization reaction conditions were reliable.

Sample processing method

Accurately weigh 18.28 mg of Chinese musk sample dried under reduced pressure to constant weight in a phosphorus pentoxide dryer, and grind 1.5 mL of EP tube, add 1 mL without Water ethanol, let stand at room temperature for 60 min, centrifuge at 13000 r/min for 10 min, gently pour out the supernatant to another EP tube, and set aside; add 1 mL of absolute ethanol to the residue again, leave it at room temperature for 60 min, and operate as above. So, repeat the extraction four times, collect the supernatant separately, accurately absorb 200 μL from each extract, place it in a 5 mL volumetric flask, derivatize according to "2.2.3"; take another musk sample 10.61 mg, In a 5 mL volumetric flask, direct derivatization was performed; all samples were tested under the chromatographic conditions of "2.1". Directly derivatized musk's unit mass peak area is 100%, and the extraction recovery rate of the extract is calculated, as shown in Figure 7. As a result, after the room temperature ethanol extraction [1], the first extraction rate was about 85% of the direct measurement, indicating that the extraction was incomplete; the direct derivatization method was more appropriate.

Method validation

Specificity test: Weigh an appropriate amount of musk sample from China and Mongolia and a certain volume of musk reference solution, derivatize according to "2.2.3", and measure according to the chromatographic conditions under "2.1". As a result, the chromatographic peak retention time of the muscone in the sample chromatogram was consistent with that of the corresponding control, and the baseline separation was achieved. The separation of the main peak from other surrounding peak was greater than 4, and the theoretical plate number was about 4000, indicating that the chromatographic conditions were more specific. Goods, can be applied to sample testing requirements, see Figure 8.

Linear relationship

Investigate a certain volume of muscone reference solution, and place it in a 5 mL volumetric flask, derivatize according to "2.2.3" and make the final concentration reach 30.00, 20.00, 10.00, 4.00, 1.00, 0.20, 0.10, 0.04 μg•mL^{-1} respectively. According to the chromatographic conditions under "2.1", with the muscone peak area (Y) as the ordinate and the solution concentration (X) as the abscissa. For the standard curve, the regression equation (n=8) is obtained: Y=1.279 × 105X-3.389 × 103, r=0.999 9. The results showed that the linear relationship of muscone in the range of 0.04-30 μg•mL^{-1} was good.

Repeatability test

Accurately absorb 20.0 μL of the muscone reference solution with a concentration of 1.0 mg•mL^{-1} for 5 parts and accurately weigh the phosphorus pentoxide dryer to reduce the weight of the Chinese musk samples 10.61, 9.95, 10.03, 10.78, 9.57 mg (strained through 40 mesh standard sieve), placed in 5 mL volumetric flasks, derivatized according to "2.2.3" and detected under chromatographic conditions under "2.1". As a result, the area RSD of the muscone reference substance was 1.60%; the unit mass area RSD of the musk sample with the same batch derivatization was 4.12%; indicating that the repeated derivatization precision of the reference substance and the musk sample was good.

Determination of the minimum detection limit

Dilute the reference solution into a series of concentrations, derivatize according to "2.2.3", and measure the chromatographic conditions under "2.1" to determine the peak area value, with baseline noise of 3. The concentration corresponding to the double (S/N) peak area is the lower limit of detection; 5 times the lower limit of detection (15 times of baseline noise) is regarded

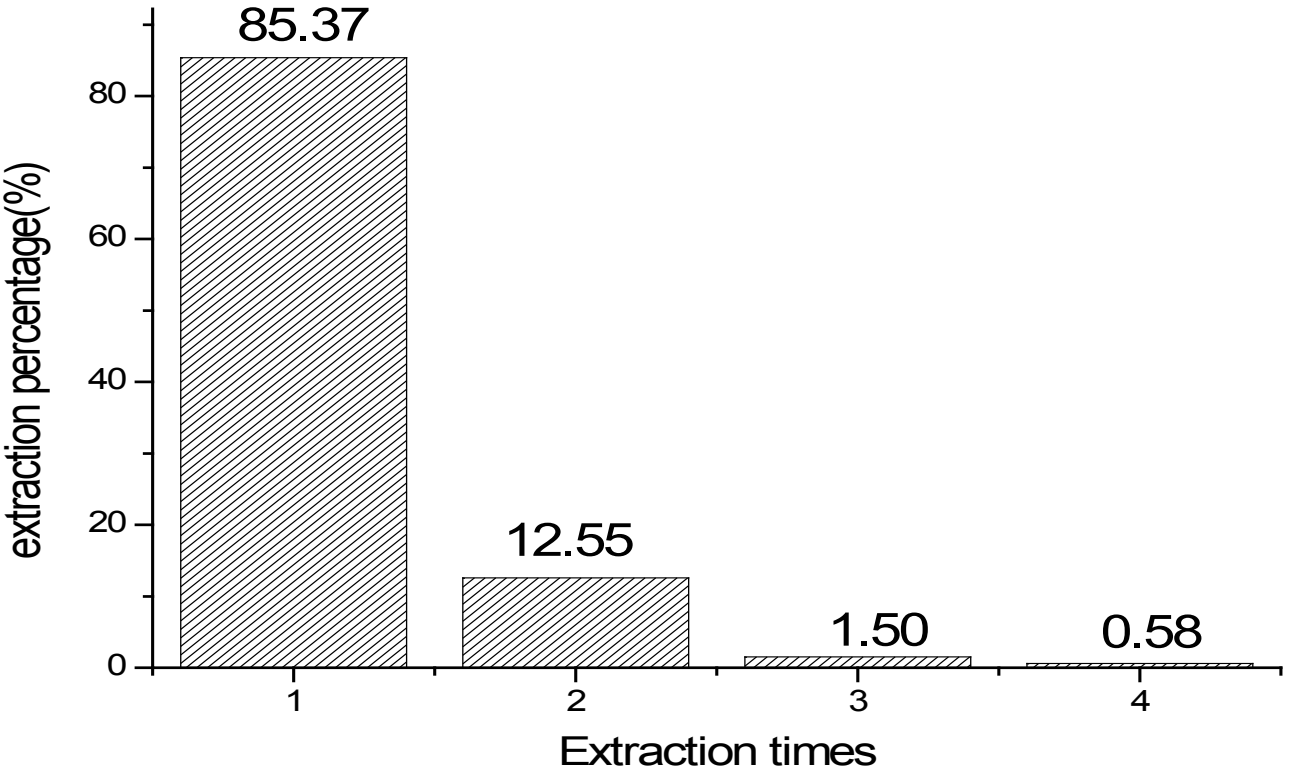


Figure 7: Influence of sample extraction.

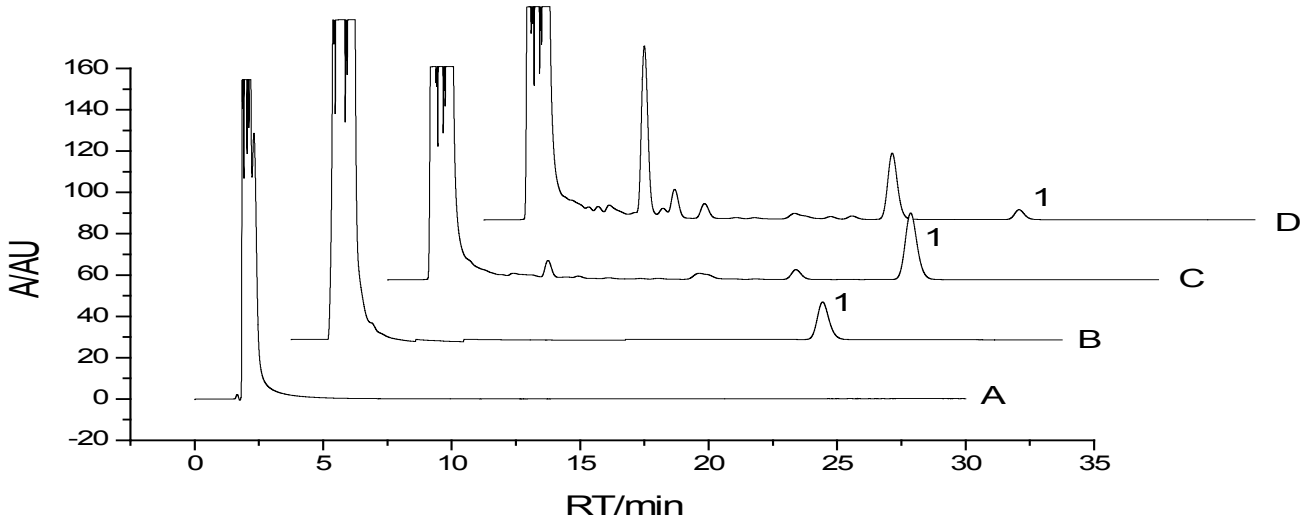


Figure 8: HPLC chromatograms. Keys: A: Derivatization reagent; B: Muscone standard; C:Musk sample/china; D: Musk sample / Mongolia; 1-muscone.

Table 4: The determination results of recovery test (n=9).

Sample conc. /μg•m L^{-1}	Standard conc. /μg•m L^{-1}	Measured conc. /μg•m L^{-1}	Recovery /%	Mean /%	RSD /%
8.56	1.00	9.57	100.65		
8.56	1.00	9.57	100.65	100.32	0.58
8.56	1.00	9.56	99.65		
8.56	10.00	18.33	97.67		
8.56	10.00	18.32	97.57	98.37	1.32
8.56	10.00	18.55	99.87		
8.48	20.00	28.07	97.97		
8.48	20.00	28.11	98.17	98.38	0.57
8.48	20.00	28.28	99.02		

as the lower limit of quantitation. As a result, the lower limit of detection of muscone was 0.005 μg•mL^{-1}, and the lower limit of quantification was 0.025 μg•mL^{-1}.

Derivatization stability

Take 1 part of the muscone reference substance and the musk sample after derivatization under "2.2.3", place it in the autosampler (23 - 28°C) for 7 days. The chromatographic conditions under 2.1" were analyzed periodically, and each sample was tested 5 times in 7 days. As a result, the peak area RSD of the muscone reference substance was 0.96%; the peak area RSD of the musk sample was 0.75%, indicating that the sample after derivatization was stable.

Sample recovery rate

Determination of precision extraction of 0.10 μg•mL^{-1} muscone reference solution 50 μL three times, 1.00 μg•mL^{-1} muscone reference solution 50, 100 μL each three, Place the 5 mL volumetric flasks separately, add the derivatized musk sample solution of known concentration, repeat the derivatization according to "2.2.3", and add the sample solution to the volume. Determine the chromatographic conditions under "2.1". Calculate the recovery rate (the sample concentration is corrected according to the actual added volume), see Table 4. Results were the average recoveries of muscone at high, medium and low concentrations ranged from 98.37 to 100.32%, and the RSD ranged from 0.57 to 1.32%.

Sample determination

According to "2.2.3", 3 batches of musk samples (including Chinese domestic batches, batch number 75541201; Mongolian domestic batches of 2 batches, all in bulk, batch number 201801, 201802) were processed according to the established HPLC method. Chromatogram, the content of 3 batches of samples was calculated by the external standard method of muscone peak area. As a result, the muscone content of the musk samples in China was 0.95% and the RSD was 4.08%. The muscone content of the musk samples in Mongolia was 0.15% and 0.32%, respectively, and the RSD was less than 4.14%.

DISCUSSION

Preparation of derivatization reagents

In the preparation of DNPH derivatization reagent, there was not much difference between hydrochloric acid and sulfuric acid in the type of acid, but the reaction in glacial acetic acid was'nt obviously complete, which may be related to the low degree of dissociation of acetic acid see Figure 3. With the increase of the amount of strong acid, the content of muscone derivative had a significant increase and then decrease; the reason for this phenomenon may be related to the moisture content of the reaction medium. For example, sulfuric acid has strong dehydration ability. When the

concentration is high, the water content of the reaction medium is increased due to dehydration of ethanol, and the water content of the reaction medium is increased. Because the dehydration reaction requires an anhydrous environment, too much water in the medium forces the reaction to reverse transfer.

In addition, the solubility of DNPH in every 100 mL of acidic ethanol was about 200 mg, and the dissolution was relatively slow. After the new solution was sonicated, the visual observation was clear, but a large amount of undissolved crystals were observed under the microscope. After leaving it at room temperature for 12 hours, the microcrystals gradually dissolved and disappeared. Therefore, the new solution must be placed overnight and filtered with a 0.2 μm microporous membrane for safer use, otherwise it will have a greater impact on accuracy.

Effect of reaction temperature

In the derivatization of muscone, the effect of temperature is very obvious see Figure 6 and Table 3. As the temperature rises, the content of the reaction product is basically reflected in an S-type change trend. This may be related to the following factors: when the temperature is below 40°C, the reaction substrate molecules in the medium move slowly, the probability of contact with each other decreases, and the dehydration process requires endotherm, so the reaction is incomplete; when the temperature is 40°C-65°C. When the above two factors are combined, the reaction will gradually be strengthened; when the temperature reaches 60°C-65°C, a relatively gentle zone appears with the exhaustion of the reaction substrate; when the temperature is raised to 70-80°C. Due to the boiling point of the medium (78°C), the amount of evaporation of ethanol will increase sharply, and the volatile muscone and hydrochloric acid will also be lost, and the content will decrease. It can be seen that the reaction temperature has an important influence on the detection sensitivity. As far as the detection sensitivity is concerned, whether or not the reaction can be quantified after the reaction reaches equilibrium at any one temperature remains to be investigated.

Effect of sample processing methods

The Chinese Pharmacopoeia 2015 edition, the gas chromatographic method [1] sample treatment, using the anhydrous ethanol extraction method, this paper was also written according to this method for the musk samples of ethanol extraction experiments. The results showed that the method had a single extraction rate of about 85% for muscone and 99% after three extractions see Figure 7. The direct derivatization of the musk samples showed that the content exceeded the sum of three extractions of ethanol. Therefore, the pre-extraction mode was abandoned on the sample processing and the sample was directly derivatized.

CONCLUSION

The established pre-column derivatization HPLC method of muscone has the advantages of accuracy and reproducibility and can be used for quantitative determination of muscone in natural musk.

CONFLICT OF INTERESTS

There are no conflicts of interests.

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