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Enzymatic hydrolysis and detection of 3-Monochloropropane-1,2-diol esters

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Abstract

There are more and more studies on the detection method of 3-chloro-1,2-propanediol fatty acid esters (3-MCPD esters) at present. By comparing these methods for the determination of 3-MCPD esters. Indirect methods, which determine total amount of 3-MCPD after hydrolysis of the esters, have an advantage over direct methods. The existing indirect methods, however, may yield unreliable results or require long hours of alkaline methanolysis. In contrast, the Indirect enzymatic hydrolysis method has mild conditions and more accurate results. In this study, we developed a reliable and rapid indirect method for determinations of 3-MCPD esters. 3-MCPD esters was enzymatized to 3-MCPD by indirect enzymatic hydrolysis method, and the conditions of enzymatic hydrolysis were optimized, the content of 3-MCPD after enzymatic hydrolysis was detected by gas chromatography-mass spectrometry (GC-MS) and the yield was calculated. Finally, the optimum conditions for enzymatic hydrolysis of 3-MCPD esters were determined. According to the optimal enzymatic hydrolysis condition, the contents of 3-MCPD esters in four food oils were determined. The method is simple and sensitive, and can meet the requirement of 3-MCPD esters detection in general oils.

Enzymatic hydrolysis and detection of 3-Monochloropropane-1,2-diol esters

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Abstract

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Keywords : 3-MCPD esters; 3-MCPD; Indirect enzymatic hydrolysis method; GC-MS

Introduction

The contamination of 3-MCPD esters is one of the hot issues in the field of food safety that have been disputed internationally in recent years[1].The formation of 3-MCPD esters is directly related to the chemical refining of synthetic oil products, It has been confirmed that 3-MCPD esters are found in hot processed foods derived from grains, fish and meat products, coffee, nuts, potatoes and edible oils[2-4]. 3-MCPD esters are hydrolyzed in vivo by intestinal pancreatic lipase to produce free 3-MCPD, 3-MCPD has extremely obvious renal toxicity and high genotoxicity, which has been strictly classified as 2B carcinogen by the International Cancer Organization Working Group (IARC) of the United Nations[5-6].

3-MCPD esters detection mainly has the direct determination method and the indirect determination method. By comparing these methods for the determination of 3-MCPD esters. Direct methods based on LC-MS/MS[7–9]are available in literature, however, the great complexity of 3-MCPD esters may lead to the determination of up to several tens of compounds in one sample for which a respective number of internal standards are required[10]. This contributes to make the procedure generally expensive, but the advantages of direct methods are the lack of derivatization step and the possibility to get direct information on the content of individual esters and for these reasons they found wide application in the field[11]. Indirect methods for the determination of 3-MCPD esters in samples requires a complex pretreatment process with many steps in advance, and the change of conditions in each experimental step will easily affect the accuracy of the final detection results, the methods are based on derivatization, transforming 3-MCPD esters in free 3-MCPD, and

they include several stages such as transesterification, neutralization and derivatization steps before quantification, using NaOH for transesterification, sodium bromide for neutralization and phenylboronic acid for derivatization[12]. But in the alkaline catalyzed indirect method, if the alkaline hydrolysis reaction time conditions and salting-out conditions or temperature are not well controlled, a part of 3-MCPD may be eliminated or extra produced, thus making the determination result small or large. Acid catalyzed indirect method is more stable and durable, but relatively time-consuming. In contrast, the indirect enzymatic hydrolysis method has mild conditions and more accurate results.

At present, there are more and more studies on the determination of 3-MCPD esters by indirect enzymatic hydrolysis method. However, there are no study have reported the optimal conditions for enzymatic hydrolysis of 3-MCPD esters. In this work, 3-MCPD esters was enzymolized to 3-MCPD by indirect enzymatic hydrolysis method, and the conditions of enzymatic hydrolysis were optimized, GC-MS was used for detection and calculation of yield, then the optimum enzymatic conditions were determined. Finally, the actual samples were detected by indirect enzymatic hydrolysis method. The method is simple, reliable, and rapid, and is suitable for a routine quality control of fats and oils[13].

Experimental Procedures

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2.1. Reagents

As the ester-form standard reagent, 3-MCPD dioleate (purity 99.8%) was purchased from Wako Pure Chemical (Osaka, Japan). As the free-form standard reagents, 3-MCPD (purity 98%) was purchased from Macklin Biochemical Co. Ltd (Shanghai, China, *www.macklin.cn),*3-MCPD-d_5 (purity 98.0%) from Larodan AB (Sweden,*www.larodan.com).* Phenylboronic acid (PBA) (purity 95%) was purchased from Sigma-Aldrich (USA,*www.sigmaaldrich.cn).*Lipase from Candida rugosa was purchased from Sigma-Aldrich. Lipase from Candida sp. and lipase from Thermophila sparsiformis was purchased from Shanghai Aladdin Biochemical Technology Co. Ltd (Shanghai, China,*www.aladdin.cn).*

A 30% (w/v) sodium bromide aqueous solution was prepared and adjusted pH with citric acid and disodium hydrogen phosphate aqueous solutions. The lipase was dissolved with this solution to prepare an aqueous solution of sodium bromide containing 150 U/mL lipase. A PBA solution was prepared by dissolving 0.25 g PBA in 10 mL water/acetone mixed solution (1:19, v/v).

1 mg of 3-MCPD esters standard was weighed, dissolved in ethyl acetate, transferred to a 10 mL volumetric flask, volumetric to calibration with ethyl acetatel. For the standard stock solutions, 300 μg/mL ethanol solutions were prepared for 3-MCPD and 200 μg/mL ethanol solutions were prepared for 3-MCPD-d_5 as internal standard stock solutions. These stock solutions could be stored at [?] 20 for at least three months. On the day of the study, the standard stock solution was diluted with the 30% sodium bromide aqueous solution to prepare 1.5 μg/mL standard solutions. Additionally, 2.0 μg/mL internal standard solutions was prepared by combining the internal standard stock solutions and diluting it with the 30% sodium bromide aqueous solution.

2.2. Oil samples

As oil samples, Palm oil, rapeseed oil and soy sauce were purchased in supermarkets. Samples were spiked with ester standards and dispensed into glass vials, and stored in the dark at room temperature.

2.3. Equipment

Electronic analytical balance (Shanghai Minqiao Precision Scientific Instrument Co. Ltd), a centrifuge (Model SC-3614; Anhui, China), laboratory pH Meter (Ohaus Instruments Co. Ltd), a vortex mixer (SCI-VS; SCILOGEX, USA), a GC-MS (Agilent 7890/5975C-GC/MSD; Agilent Technologies, USA), a constant-temperature shaker (SHA-B; Guohua company, China)

2.4. Enzymatic hydrolysis of 3-MCPD esters standard

1 mg of 3-MCPD esters standard was weighed and dissolved in a 10 mL test tube with ethyl acetate. After mixing, 20 uL of the above solution was put into a 5 mL test tube. After adding 3 mL of the 30% sodium bromide aqueous solution containing 150 U/mL lipase, the mixture was shaken by a high-speed shaker for 30 min at room temperature to hydrolyze the esters. The mixture was then heated in a water bath at 80 for 10 min. The mixture was cooled to room temperature, and 50 μL of 2.0 μg/mL internal standard mix was added. Next, 3 mL of hexane was added and the tube was vortexed for 10 s. The aqueous layer was transferred to a new test tube, to which another 3 mL of hexane was added and the tube was vortexed again for 10 sec. After the hexane layer was removed, 3-MCPD in the aqueous layer and the deuterated free form internal standards were derivatized by adding 100 μL of PBA solution and 3 mL of hexane, and shaking at room temperature for 5-10 min. The organic layer was then transferred to a new test tube containing sodium sulfate and concentrated to approximately 0.5-0.8 mL under a stream of nitrogen. After filtration through a 0.2 μm membrane filter, the sample was subjected to GC-MS analysis.

2.5. Procedures for analysis of3-MCPD esters

In a 10 mL test tube, 0.1 g of oil sample was dissolved in 200–500 μL of isooctane. After adding 3 mL of 30% NaBr aqueous solution containing 150 U/mL lipase, the mixture was shaken by a high-speed shaker for 30 min at room temperature. The test tube was heated for 10 min in an 80 water bath. The test tube was cooled to room temperature, and 50 μL of 2.0 μg/mL internal standard mixed solution was added. Next,

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3 mL of hexane was added and shaken for 10 min. The resulting aqueous layer was transferred to a new test tube. Another 3 mL of hexane was added and the tube was shaken for 10 sec; the resulting hexane layer was subsequently removed. A 100 μL PBA solution was added to the aqueous layer, and the mixture was agitated with a vortex mixer for 10 sec. Next, 3 mL of hexane was added and shaken for 10 sec. The resulting organic layer was transferred to a new test tube and concentrated to approximately 0.5–0.8 mL using a stream of nitrogen. After filtration through a 0.2 μm membrane filter, the samples were subjected to GC-MS analysis.

2.6. GC-MS Analysis

A GC capillary column was used with a (5%-phenyl)-methylpolysiloxane liquid phase, 30 m length, 0.25 mm internal diameter, and 0.25 μm film thickness. The carrier gas was helium at a fixed flow rate of 1.2 mL/min. The sample was injected in splitless mode at 250. The column oven temperature was held at 60 for 1 min, then raised to 150 at 10/min, 180 at 3/min, and finally 300 at 30/min, before being held at 300 for 8 min (total run=32 min). MS was performed in positive electron ionization mode with an ion source temperature of 230. Quantitative and qualitative analyses were performed by selective ion monitoring (SIM) using ions at m/z 147 and 196 for the 3-MCPD derivative, 150 and 201 for the 3-MCPD-d5 derivative.

Results and Discussions

3.1. Validation of lipase hydrolysis

To verify that the lipase acted only on 3-MCPD esters, three controls were used. The comparison of lipase action on 3-MCPD and lipase action on 3-MCPD esters, the comparison of lipase action on 3-MCPD esters and lipase no action on 3-MCPD esters, the comparison of pure solution of 3-MCPD, lipase action on 3-MCPD and pure solution of lipase. As seen in the chromatogram, comparison between the effect of lipase on 3-MCPD and the effect of lipase on 3-MCPD esters (Fig. 1), the peak time, mass spectrogram information and ratio in qualitative ion map of the two were similar, indicating that the same substance was finally obtained, that is, the enzymatic hydrolysis was successful.

Comparison between the effect of lipase action on 3-MCPD esters and lipase no action on 3-MCPD esters (Fig. 2). 3-MCPD is produced by the action of lipase on 3-MCPD ester, but the lipase does not act on the 3-MCPD ester does not produce the 3-MCPD, and 10.705 min is peak time of 3-MCPD esters. This indicated that the enzymatic hydrolysis was successful.

Comparison among pure solution of 3-MCPD, lipase action on 3-MCPD and pure solution of lipase (Fig. 3). The comparison of the three results shows that, the response of pure solution of 3-MCPD and lipase action on 3-MCPD is almost the same. However, the pure solution of lipase do not produce 3-MCPD peak, indicating that the lipase can not act on 3-MCPD. Therefore, 3-MCPD produced by lipase action on 3-MCPD esters is successful, and has nothing to do with the addition of 3-MCPD internal standard during the experiment.

3.2 Selection of

lipases

To select the lipase suitable for hydrolysis of 3-MCPD esters, the recovery of 3-MCPD esters was also analyzed using lipase from Candida rugosa , lipase from Candida sp. and lipase from Thermophila sparsiformis at 150 U/mL. All lipases were prepared at 150 U/mL in 30% NaBr buffer, and a 0.5 h lipolysis was performed at the optimal pH and temperature proposed by the manufacturer in triplicate. As shown in Table 1, 3-MCPD esters could not be enzymatically decomposed by lipase from Thermophila sparsiformis. Among the lipases tested, lipase from Candida sp. gave the highest recovery rate for 3-MCPD. These results suggested that lipase from Candida sp. had higher substrate specificity for hydrolysis of 3-MCPD esters than other lipases tested.

3.3 Optimization of hydrolysis condition for the lipase from Candida sp.

Firstly, pH were studied in the optimal range proposed by the manufacturer (5 [?] pH [?]9). we studied the

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effect of different pH of the 3-MCPD on the yield. Hydrolysis was performed by adding 3 mL of 30% (w/v) sodium bromide aqueous solution (pH 5.0, pH 6.0, pH 7.0, pH 8.0, pH 9.0) containing 150 U/mL of lipase. As shown in Fig.4, among the tested pH, the yield of 3-MCPD was the highest when pH was 5.0, achieving acceptable results at all three levels.It has been shown that the change of pH value will cause the change of enzyme conformation, leading to the change of enzyme catalytic activity[14]. The results show that the optimal pH of lipase from Candida sp. is 5. What is more, the lipase from Candida sp. is inactivated under alkaline conditions.

Secondly, to determine optimal lipase concentration in the hydrolysis buffer, we analyzed 3-MCPD esters standard solution at 0.1 mg/mL by using the hydrolysis buffer containing a concentration of lipase in the range between 90 and 210 U/mL were studied in triplicate spanning, and by adding 3 mL of 30% (w/v) sodium bromide aqueous solution (pH 5.0) after completion of the hydrolysis step of 30 min. Good yield for 3-MCPD (95%) were obtained when the lipase concentration was 150 U/mL at all concentrations tested (Fig.5). From the result, it was decided to use 150 U/mL of lipase from Candida sp. in the hydrolysis buffer.

Thirdly, the effects of enzymatic hydrolysis time were studied by varying from 10 min to 60 min. As shown in Fig.6, the yield for 3-MCPD increased and then decreased with the increase of enzymatic hydrolysis time. It was suggested that optimum time for enzymatic hydrolysis is 30 minutes. And with the increase of time, the lipase activity decreased.

Lastly, in order to find appropriate temperature for enzymatic hydrolysis of 3-MCPD esters, different temperatures such as 15, 20, 25, 30 and 35 were studied in triplicate spanning. As shown in Fig.7, as the temperature rises, the yield for 3-MCPD increased first, then decreased, When the hydrolysis temperature reached 25, the lipase activity reached the highest value, After that, the higher the temperature, the smaller the yield for 3-MCPD. It can be seen that the lipase is sensitive to temperature, and the activity of the lipase can be increased by increasing the hydrolysis temperature appropriately. On the other hand, the high hydrolysis temperature will reduce the lipase activity and reduce the service life of the lipase.

3.4 Feasibility Study

3.4.1 Standard curve, regression equation and detection limit

The 3-MCPD standard solution was used for derivatization to make the standard curve, and then the sample was spiked with the standard solution to determine the content of 3-MCPD ester in edible oil. Due to the esterification reaction of 3-MCPD to free 3-MCPD, the structure characteristics of 3-MCPD itself lead to the shortcomings of the chromatographic peak width and low sensitivity in the direct determination of 3-MCPD, so the test will be further derivatization of 3-MCPD[15].

The 3-MCPD standard solution of 10, 50, 100, 200, 400 ug was diluted with the prepared standard stock solution,operate according to the steps of 2.4 adding derivatization reagent (PBA), and test according to the GC-MS Analysis conditions of 2.6. The standard curve was drawn with the amount of 3-MCPD (X) as abscissa and the peak area (Y) as ordinate. At the same time, 3 times SNR was used to determine the minimum detection limit. Standard curve of 3-MCPD is showed in Fig.8. The linear range, regression equation, correlation coefficient and detection limit of this method are shown in Table 2.

3.4.2 Precision and recovery rate

Three levels of 3-MCPD ester recovery experiments were carried out with vegetable oil as substrate, Repeat six times for each spiked level. Under the optimal enzymatic hydrolysis conditions, the recovery of 3-MCPD was 92.1%-101.4%, the relative standard deviation (RSD) of 3-MCPD ester was 3.8%-4.3%, the results are shown in Table 3. According to the regression equation, detection limit, recovery rate and other methodological parameters, The method has the characteristics of high sensitivity, good specificity and high precision for the determination of 3-MCPD esters in vegetable oil. It can satisfy the purpose of determining the total 3-MCPD esters in vegetable oil.

3.5 Determination of 3-MCPD esters in oil samples

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Soybean oil, rapeseed oil, palm oil and peanut oil were detected by gas GC-MS, which was established by enzyme-hydrolyzed sample pretreatment method. According to the experimental method of 2.5, each sample was measured six times in parallel to take the average value, the content of 3-MCPD ester was calculated according to the peak area and linear regression equation obtained by GC-MC detection. The contents of 3-MCPD ester in palm oil, peanut oil, rapeseed and soybean oil were as follows: 5.52, 3.02, 2.87 and 2.73 mg/kg.

Conclusion

In this study, the optimal conditions for enzymatic hydrolysis of 3-MCPD esters were obtained by single factor test as follows: lipase from Candida sp. was selected, enzyme concentration was 150 U/mL, the enzymatic hydrolysis temperature was 25, the enzymatic hydrolysis time was 30 min, and the pH was 5. Secondly, the standard curve of the 3-MCPD standard solution was drawn, and then the edible oil sample was processed before enzymatic hydrolysis according to the optimal enzymatic hydrolysis conditions, derived with PBA, and finally detected by GC-MC. The content of 3-MCPD ester in four kinds of vegetable oil was obtained by combining the standard curve, and the content of 3-MCPD ester in food oil could be detected simply and quickly.

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Ting Xu: Project administration, Resources, Writing- review & editing. **Min Fang:** Methodology, Software, Writing-original draft. **Qingru Zeng:** Software, Investigation. **Xin Liu:**Supervision and Data curation. **Shensheng Xiao:** Validation and Investigation. **Xiaoming Jiang:** Resources, Writing- review & editing. **Yuepeng Lu:** Validation and Investigation.**Zhiyong Gong:** Investigation and Funding acquisition. All authors contributed to and approved the final draft of the manuscript.**Yonggui Gao:** Reviewing and editing.

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