Thesis for the Degree of Master of Engineering

Development and Validation of Simultaneous Analysis for Maltooligosaccharides and Isomaltooligosaccharides

by

Junho Yang

Department of Food Science and Technology

The Graduate School

Pukyong National University

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(말토올리고당과 이소말토올리고당의 동시분석법 개발 및 검증)

Advisor: Prof. Ji-Young Yang

by
Junho Yang

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Junho Yang

Approved by:

(Chairman) Yang-Bong Lee. Ph.D.

(Member) Young-Mog Kim. Ph.D.

(Member) Ji-Young Yang. Ph.D.

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Contents

요 약 iii

I. Introduction 1

II. Materials and Methods 6

1. Materials 6

2. Standard preparation 6

3. Instrumentation 7

4. Chromatographic condition 7

5. Method validation 8

6. Statistical analysis 10

III. Results and Discussion 11

1. Optimization of analysis method for maltooligosaccharides and isomaltooligosaccharides 11

2. Validation of the method for analyzing maltooligosaccharides and isomaltooligosaccharides 13

3. Linearity of the method for analyzing maltooligosaccharid
es and isomaltooligosaccharides 14
4. Detection and quantitation limits of the method for analyzi
ng maltooligosaccharides and isomaltooligosaccharides..... 18
5. Accuracy of the method for analyzing maltooligosacchari
des and isomaltooligosaccharides 19
6. Precision of the method for analyzing maltooligosacchari
des and isomaltooligosaccharides 21
IV. Conclusion 23
V. Summary 25
VI. References..... 27

말토올리고당과 이소말토올리고당의 동시 분석법 개발 및 검증

양 준 호

부경대학교 대학원 식품공학과

요 약

Charged aerosol detector (CAD)는 발색단이 없거나 자외선을 흡수하지 않는 화합물을 감지 할 수 있기 때문에 응용 범위가 넓고, 광학적 또는 이온화 특성에 크게 의존하지 않으며, 감도와 재현성이 우수하고, 경사 용출 호환성과 같은 장점이 있다. 말토올리고당(Maltooligosaccharides, MO)과 이소말토올리고당(Isomaltooligo-saccharides, IMO)은 식품으로서 다양한 기능을 가지고 있다. 그러나 MO 및 IMO 를 분석하는 방법은 확립되지 않았으며, 정확한 정량분석에 제한이 있다. 따라서, 본 연구는 HPLC-CAD 를 사용하여 MO (DP2-6) 및 IMO (DP2-6) 분석을 위한 동시 분석 방법을

수립하고 검증을 통하여 신속하고 정확한 분석법 개발을 목표로 하였다. 아미노컬럼(HILICpak VG-50 4E) 및 CAD 를 분석에 사용하였다. 이동상으로는 아세토니트릴(acetonitrile, ACN)과 물로 구성되었으며, 기울기 용리 조건으로는 ACN 을 0 분에서 50 분까지 80%에서 66% 감소되고, 50 분에서 60 분까지 66%에서 60%로, 60 분에서 60.10 분까지 60%에서 80%로, 60.10 분에서 65 분까지 80%로 변화되도록 하였다. 유속은 0.7 mL/min, 컬럼 오븐 온도는 40° C, 시료 주입량은 5 μL 로 분석조건을 설정하였다. CAD 의 분석 조건은 증발기 온도는 40℃, 데이터 수집 속도는 5 Hz 로 설정하였다. 직선성은 상관 계수(R^2)로 표현하였으며, 모두 0.99 이상을 나타내었다. 검출한계(Limitied of detection, LOD)와 정량한계(Limited of quantitation, LOQ)는 각각 3-12 ppm 과 8-32 ppm 이었다. 일내 및 일간 정확도는 93.6±2.3-109.5±0.4%로 나타내었으며 실험간에 유의적인 차이가 없었다(p<0.05). 정밀도는 1.70-4.07%로 나타내었다. 새롭게 개발된 방법은 검증절차를 통해 MO 와 IMO 의 동시 분석에 적합하였다.

I. Introduction

Charged aerosol detector (CAD) is a detector that converts an electric charge into a signal by using an electrometer to make the object analyze in an aerosol state and then to take on an electric control. The CAD process converts the analyte into particles. The effluent from HPLC column is sprayed with nitrogen gas and dried to generate particles. The particles move to the collector, where a highly sensitive electrometer measures the charge-producing direct proportional signal to the analyte amount (Dixon., 2002). Besides CAD, refractive index detector (RID) and evaporative light scattering detector (ELSD) are typically used for sugar analysis (Isabel et al., 2013). However, RID has limitations in the accurate analysis due to its high sensitivity to ambient temperature and flow rate changes and low signal to noise ratio (Zhou et al., 2014). Since CAD can detect compounds that do not have a chromophore or absorb ultraviolet rays, it has a wide range of applications. It does not depend heavily on optical properties or ionization properties has excellent sensitivity (about 10 times ELSD) and

reproducibility and is suitable for gradient elution. Advantages have been reported (Eom et al., 2010; Inagaki et al., 2007).

Maltooligosaccharides (MO) are straight-chain oligosaccharides in which 3 to 10 glucose are linked by α-(1→4) bonds. It has a low-low taste and low viscosity, the anti-aging inhibitory effect of starch, and excellent moisture retention. It is used to improve physical properties in frozen bread dough, frozen food, confectionery, bakery, sediment, soy milk products, etc. (Lee et al., 1998). Representative MOS include maltose, maltotriose, maltotetraose, maltopantaose, and maltohexaose (Fig. 1). Isomaltooligosaccharide (IMO) is a branched oligosaccharide in which glucose molecules have α-1, 6 bonds produced by the transfer reaction of maltose or MO, a sugar transfer enzyme. Representatively, isomaltose, isomaltotriose, isomaltotetraose, isomaltopentaose, isomaltohexaose (Fig. 2), and the like. IMO's physiological functions are slowly decomposed by intestinal enzymes to lower the glycemic index (Sorndech et al., 2018), improve immune function, increase the bioavailability of some minerals, and promote the metabolism of triglycerides (Qiang et al., 2009). Effects such as improving cholesterol have been reported (Yen et al., 2011).

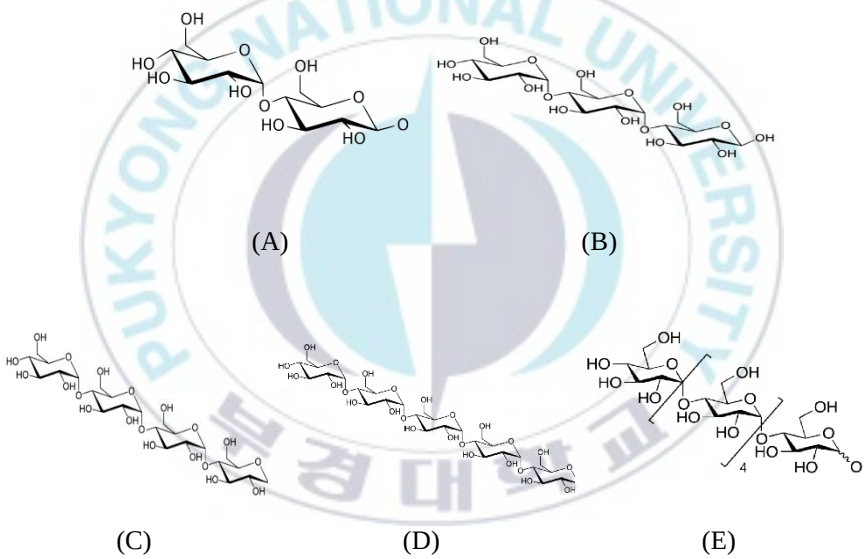

Fig. 1. Chemical structures of maltose (A), maltotriose (B), maltotetraose (C), maltopentaose (D), and maltohexaose (E).

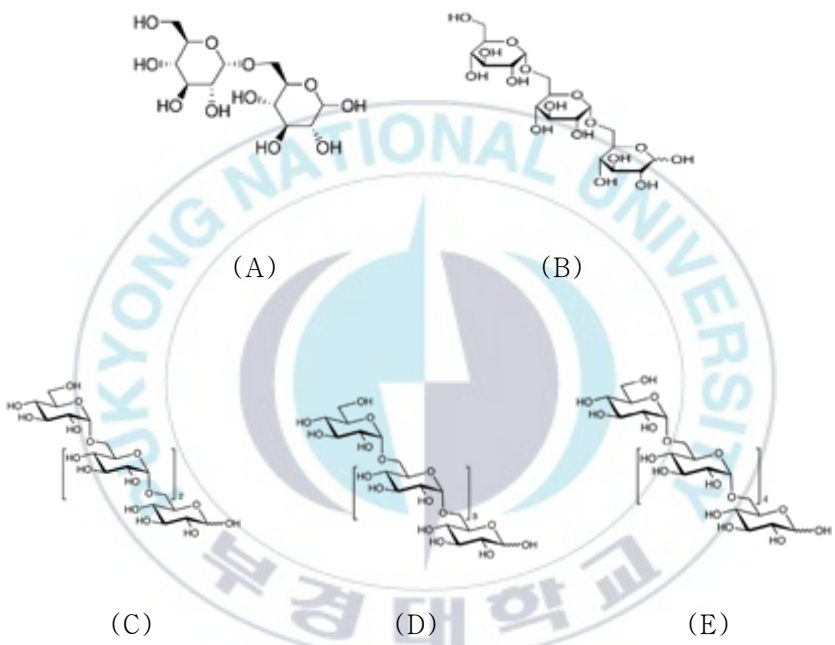

Fig. 2. Chemical structures of isomaltose (A), isomaltotriose (B), isomaltotetraose (C), isomaltopentaose (D), and isomaltohexaose (E).

Also, IMO is widely used in food due to its features such as heat stability, low viscosity, and high moisture retention. It is not fermented by yeast to be used as a sweetener for baking, alcohol, seasoning, etc (Gourineni et al., 2018). It is a trend that is attracting attention. Studies on the analysis of MO and IMO use have been reported. There is currently no analysis method to describe high performance liquid chromatography in a test method for healthy and functional foods. Besides, MO and IMO have similar chemical formulas, making simultaneous analysis difficult. Therefore, it is intended to establish an analysis method capable of simultaneous analysis of MO and IMO.

In this study, a high performance liquid chromatography-charged aerosol detector (HPLC-CAD) was used to optimize MO and IMO's simultaneous analysis. Linearity, limits of detection (LOD), limits of quantitation (LOQ), inter and intra-day accuracy and precision were investigated to validate the simultaneous analysis method.

II. Materials and Methods

1. Materials

Maltose (MO2), maltotriose (MO3), maltotetraose (MO4), maltopentaose (MO5), and maltohexaose (MO6) were purchased from Elicity Oligotech (Crolles, France). Isomaltose (IMO2), isomaltotriose (IMO3), isomaltotetraose (IMO4), isomaltopentaose (IMO5), and isomaltohexaose (IMO6) were purchased from Carbosynth (Compton, UK). HPLC-grade acetonitrile (ACN) and water were purchased from Honeywell Burdick and Jackson (Michigan, MI, USA).

2. Standard preparation

Maltooligosaccharides (maltose, maltotriose, maltotetraose, maltopentaose, and maltohexaose) and isomaltooligosaccharides (isomaltose, isomaltotriose, isomaltotetraose, isomaltopentaose, and

isomaltohexaose) sample solutions were prepared at 10,000 ppm in water. Then, each concentration flowed with water for method validation.

3. Instrumentation

Chromatographic experiments were performed on HPLC (Ultimate 3000, Thermo Scientific Inc., Waltham, MA, USA) coupled with a charged aerosol detector (Corona Veo RS, Thermo Scientific Inc., Waltham, MA., USA). Data processing was carried out with Chromelone software ver.6.8 (Dionex), and the nitrogen gas flow rate was regulated automatically and monitored by the CAD. Gas was supplied by nitrogen generator Corona 1010 (Peak Scientific, Inchinnan, UK). The nitrogen gas pressure of the CAD was adjusted to 62.1 psi.

4. Chromatographic condition

Chromatographic separation was achieved using an amino column (HILICpak VG-50 4E, Shodex, Tokyo, Japan) that is not complicated to

separate MO and IMO. Mobile phase composed of water (A) and acetonitrile (C) in gradient run from 60%C to 80% in 65 min. The flow rate was 0.7 mL/min, the column oven temperature was 40℃, and the sample injection volume was 5 μL. The operating conditions of HPLC-CAD for analysis of MO and IMO were presented in detail in Table 1.

5. Method validation

According to ICH harmonized tripartite guideline, the method validation procedure was carried out (ICH Guideline Q2 (R1), 2005). The proposed HPLC-CAD method was evaluated in terms of linearity, limited detection (LOD), limited quantitation (LOQ), precision, and accuracy. All of the validation procedure was performed in triplicate. The linearity was evaluated by preparing 10 standard mixtures of MOS and IMOS at 20 to 1000 ppm. The result was expressed by the correlation coefficient (R^2). The LOD and LOQ were calculated at the signal-noise ratio (S/N) of approximately 3:1 and 10:1, respectively. Intra and inter-day accuracy and precision were assessed by determining a mixture of MO and IMO

Table 1. HPLC–CAD (charged aerosol detector) condition of for analysis of maltooligosaccharides and isomaltooligo-saccharides

Flow	0.7 mL/min		
Column	HILICpak VG-50 4E 250 mm x 4.6 mm, 5 μm		
Oven temp	40℃		
Inj. volume	5 μL		
Sampler temp.	10℃		
Mobile phase	Multi gradient		
	min	Water	Acetonitrile
	0.00	20	80
	50.00	34	66
	60.00	40	60
	60.10	20	80
	65.00	20	80
Detector	Charged aerosol detector Evaporator temp: 40℃ Data collection rate: 5 Hz		

samples at three concentration levels on three days.

6. Statistical analysis

Statistical processing was performed using Minitab (R19) program and verified at a 95% confidence interval through one-way ANOVA analysis to confirm the significant difference in intra and inter-day accuracy experimental results.

III. Results and Discussion

1. Optimization of analysis method for maltooligo-saccharides and isomaltooligosaccharides

A representative chromatogram of MO and IMO, which obtained the advanced HPLC-CAD method, is shown in Figure 1. The optimized analysis method of MO and IMO was determined according to peak shape and degree of separation. A retention time of 15.64 min (MO2), 17.38 min (IMO2), 22.87 min (MO3), 26.82 min (IMO3), 30.92 min (MO4), 36.48 min (IMO4), 38.68 min (MO5), 43.06 min (IMO5), 44.87 min (MO6), 52.12 min (IMO6) can be observed from HPLC chromatogram in Fig. 1.

When comparing chromatograms for the simultaneous MO and IMO analysis using HPLC-ELSD, the order of sugar detection was consistent with this experiment, and selective separation was possible. Besides, each residence time is about 48 min (MO2), 50 min (IMO2), 57 min (MO3), 59 min (IMO3), 64 min (MO4), 65 min (IMO4), 68 min (MO5), 69 min

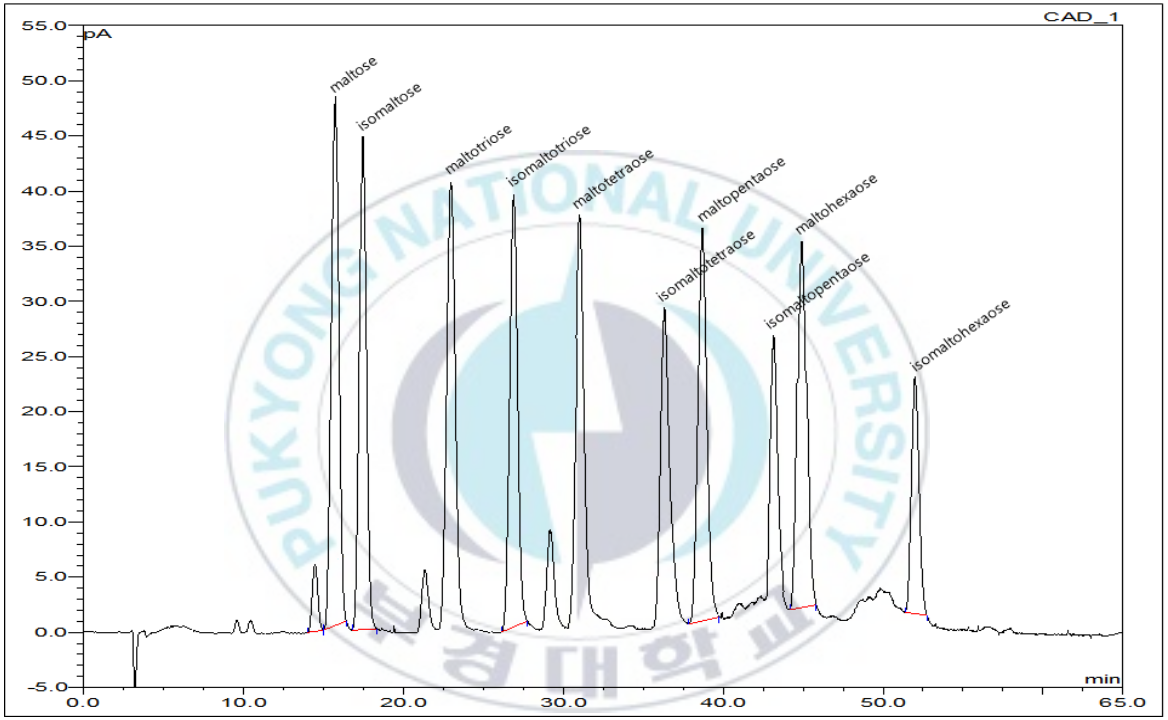

Fig. 3. HPLC–CAD (charged aerosol detector) chromatogram of maltooligosaccharides and isomaltooligosaccharides.

(IMO5), 71 min (MO6) and 72 min (IMO6) (Ko et al., 2013). In the case of maltose, the retention time-analyzed using HPLC-CAD under different analysis conditions was 34.98 min (Grembecka et al., 2014), and the retention time of maltose according to the analysis conditions of this study was 15.64 min, which was detected more quickly.

This study suggests the simultaneous MO and IMO analysis using CAD to measure the kinds and amounts of oligosaccharides.

2. Validation of the method for analyzing maltooligo-saccharides and isomaltooligosaccharides

The method validation procedure was carried out according to ICH guideline Q2 (R1) (2005). The MO and IMO analysis method was validated by evaluating the linearity range and limit of detection (LOD), limit of quantification (LOQ) and precision, accuracy. The precision and accuracy were evaluated on intra and inter-days.

3. Linearity of the method for analyzing maltooligo-saccharides and isomaltooligosaccharides

The linearity of detector response was determined by the square correlation coefficients (R^2) of the calibration curves generated by standard solutions at seven concentration levels. The calibration curves for the analytes, mixed working solutions ranging from 20 to 1000 ppm were investigated, and each working solution was injected three times.

The calibration curves and correlation coefficients of 10 MO and IMO types are shown in Fig. 4 and 5. The peak areas were plotted against the amounts (ppm) of analyte injected. The linearity range and R^2 values are presented in Table 2.

A linear equation can express responses obtained in the examined range with good R^2 values. In the analyzed concentration ranges, the CAD linearity meets the requirement of $R^2 \geq 0.99$, typically specified in method validation protocols. This result showed a correlation coefficient similar to the result of the study confirming the excellent linearity of isooligosaccharide using HPLC-ELSD (Ko et al., 2013).

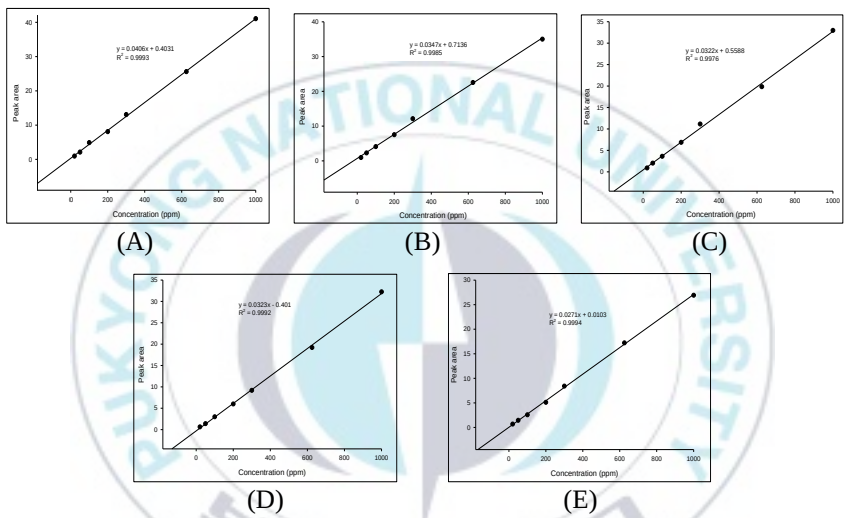

Fig. 4. Calibration curve of maltooligosaccharides by HPLC–CAD (charged aerosol detector) method.
(A, maltose; B, maltotriose; C, maltotetraose; D, maltopentaose; E, mal-tohexaose)

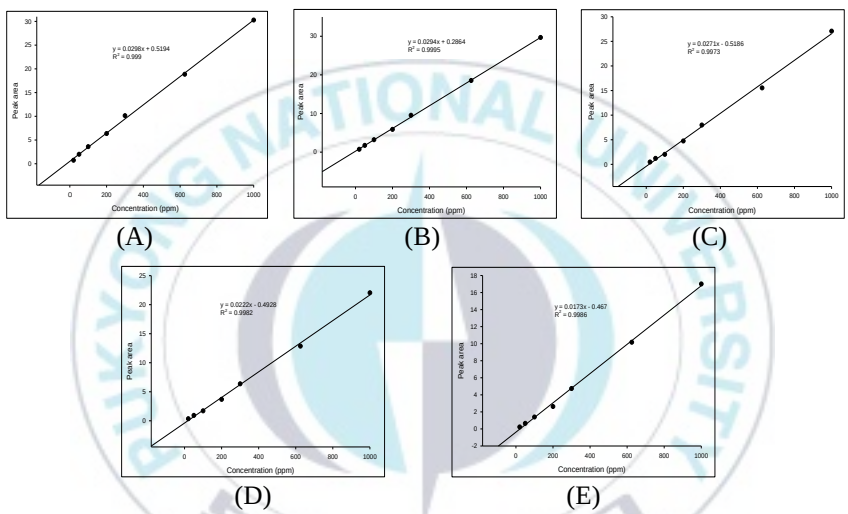

Fig. 5. Calibration curve of isomaltooligosaccharides by HPLC–CAD (charged aerosol detector) method.
(A, isomaltose; B, isomaltotriose; C, isomaltotetraose; D, isomalto-pentaose; E, isomaltohexaose)

Table 2. Linearity, detection and quantitation limits of maltooligosaccharides and isomaltooligosaccharides by HPLC–CAD (charged aerosol detector) method

DP[1)]	MOS[2)]	Regression equations	R^2	LOD[3)] (ppm)	LOQ[4)] (ppm)
2	MO2	y = 0.0407x + 0.3319	0.9994	3	8
	IMO2	y = 0.0298x + 0.5194	0.999	3	8
3	MO3	y = 0.0347x + 0.7136	0.9985	3	10
	IMO3	y = 0.0294x + 0.2864	0.9995	3	10
4	MO4	y = 0.0322x + 0.5588	0.9976	4	10
	IMO4	y = 0.0271x - 0.5186	0.9973	8	15
5	MO5	y = 0.0323x - 0.401	0.9992	4	10
	IMO5	y = 0.0222x - 0.4928	0.9982	10	16
6	MO6	y = 0.0271x + 0.0103	0.9994	10	16
	IMO6	y = 0.0173x - 0.467	0.9986	12	32

[1)] DP, degree of polymerization

[2)] MOS, maltooigosaccharides, and isomaltooligosaccharides(MO2, maltose; IMO2, isomaltose; MO3, maltotriose; IMO3, isomaltotriose; MO4, maltotetraose; IMO4, isomaltotetraose; MO5, maltopentaose; IMO5, isomaltopentaose; MO6, maltohexaose; IMO6, isomaltohexaose)

[3)] LOD, limit of detection

[4)] LOQ, limit of quantitation

4. Detection and quantitation limits of the method for analyzing maltooligosaccharides and isomaltooligo-saccharides

The detection and quantification limits for the 10 types of MO and IMO were confirmed by the signal-noise ratio (S/N ratio) for each concentration and presented in Table 2.

The estimation of detection and quantification limits was done based on the calibration curve. The LODs were determined to be in the range of 3-12 ppm, and LOQs ranged from 8-32 ppm, respectively. When comparing LOD and LOQ limit results of MO and IMO using HPLC-ELSD (Ko et al., 2013), detection sensitivity and quantitation limit tended to increase as the degree of polymerization increased. It was found that the correction factor values for MO and IMO tended to decrease as the degree of polymerization increased (Lee et al., 2004), and the sensitivity of analysis using HPLC-CAD increased as the degree of polymerization of MO and IMO increased.

5. Accuracy of the method for analyzing maltooligo-saccharides and isomaltooligosaccharides

Table 3 showed the accuracy results of 10 types of MO and IMO. The accuracy was expressed as a percentage of the means for each peak divided by the true value.

As a result of measuring intra-day and inter-day accuracy, the intra-day accuracy is 100.03±1.94-100.16±3.65%, and the inter-day accuracy is 99.88±3.63-100.17±3.49%, respectively. The values of intra and inter-day accuracy in three concentrations of MO and IMO solutions were close to 100% indicate the high accuracy of the method. In this study, there is a similarity between the attitudes expressed and the attitudes described as accuracy results (Pramod et al., 1986). There was no significant difference between the daily experimental results in 95% of the confidence groups as a result of validation by one-way batch variance (ANOVA) to confirm a significant difference in intra and inter-day accuracy for 3 days. In a similar study, one-way batch analysis of variance confirmed no significant difference between the daily experimental results at the 95% confidence

Table 3. Accuracy, precision of maltooligosaccharides and isomalto ligosaccharides by HPLC–CAD (charged aerosol detector) method

MOS[1)]	Intra-day		Inter-day	
	Accuracy (%)	Precision (RSD[2)],%)	Accuracy (%)	Precision (RSD,%)
MO2	100.03±2.08 [ab]	2.09	100.05±2.71 [ab]	2.73
IMO2	100.04±1.96 [ab]	1.97	100.04±2.28 [ab]	2.28
MO3	100.07±3.18 [ab]	3.20	100.02±1.64 [ab]	1.63
IMO3	100.08±3.07 [ab]	3.02	100.02±1.47 [ab]	1.48
MO4	100.03±1.94 [ab]	1.96	100.02±1.39 [ab]	1.41
IMO4	100.13±3.93 [ab]	3.96	100.04±2.05 [ab]	2.04
MO5	100.12±3.22 [ab]	3.29	100.01±1.39 [ab]	1.39
IMO5	100.16±3.65 [ab]	3.71	99.88±3.63 [ab]	3.71
MO6	100.06±2.25 [ab]	2.30	100.01±0.94 [ab]	0.94
IMO6	100.13±3.95 [ab]	3.85	100.17±3.49 [ab]	3.61

[1)] MOS, maltooigosaccharides, and isomaltooligosaccharides
[2)] RSD, relative standard deviation
[a] Mean±S.D (n=3)
[b] Intra and inter-day accuracy were not significantly different by one way ANOVA (p<0.05)

level (Ko et al., 2013). In this study, it is considered desirable to verify at a 95% confidence level for daily accuracy verification.

6. Precision of the method for analyzing maltooligo-saccharides and isomaltooligosaccharides

Table 3 shows the precision measurement results for 10 types of MO and IMO. The precision of the method was calculated by measuring repeatability for each compound in the MO and IMO solutions with three concentrations. The precision is expressed as a relative standard deviation (RSD, %). It is described as a measure of the variability of the results and the repeatability of the method (Shalaby et al., 2011).

As a result of measuring the intra-day and inter-day precision, the intra-day precision was 1.96-3.96%, and the inter-day precision was 0.94-3.71%. Low values described the precisions with a relative standard deviation of 5% or less. Similarly, Some research presented a relative standard deviation that proposed a method lower than 10% (Ko et al., 2013; Shalaby et al., 2011). In this study, the low precision with a relative

standard deviation of 5% or less suggests an excellent precision of the developed HPLC method.

IV. Conclusion

This study overcame the limitations of the IMO analysis method according to the current food method and developed a new analysis method to analyze quickly and accurately MO. IMO analysis limitations improved by applying a CAD detector that is highly sensitive and easy for gradient elution analysis. An amino column for easy sugar analysis is used to optimize instrument analysis conditions. The newly developed analytical method verified that the linearity, LOD, LOQ, accuracy, and precision are valid by a validation procedure.

In the results of this study, the correlation coefficient of linearity was 0.99 or more, and the accuracy value was close to 100%. The relative standard deviation of less than 5% precision suggests the excellent precision of the developed HPLC method. LOD and LOQ were expressed as 3-12 ppm and 8-32 ppm, respectively. This method was validated to be a suitable method for MO and IMO analysis.

This study's results are expected to be a technology that can quickly and accurately analyze MO and IMO. This technology enables MO and IMO

analysis in foods, which is expected to be used as necessary data for the study of MO, IMO, and other sugars.

V. Summary

Since CAD can detect compounds that do not have a chromophore or absorb ultraviolet rays, it has a wide range of applications. It does not depend heavily on optical or ionization properties, has excellent sensitivity and reproducibility, and has advantages such as gradient elution compatibility. MO and IMO have various functions as food. However, a method for analyzing MO and IMO has not been established.

This study was aimed to confirm and validate a simultaneous method for analysis MO (DP2-6) and IMO (DP2-6) using HPLC-CAD. Amino column (HILICpak VG-50 4E) and charged aerosol detector (CAD) were used for analysis. The mobile phase consisted of acetonitrile (ACN) and water.

The optimized condition was obtained at a linear gradient elution with 60-80% ACN in 65 min. The flow rate was 0.7 mL, the column oven temperature was 40℃, and the sample injection volume was 5 μL. Conditions of the detector were set to 40℃ for evaporator temperature and

5 Hz for data collection rate. The linearity of the optimized HPLC-CAD method of MO and IMO by the correlation coefficient (R^2) was 0.99 or more. LOD and LOQ were 3-12 ppm and 8-32 ppm, respectively. Intra and inter-day accuracy ranged from 93.6±2.3-109.5±0.4%, and precision ranged from 1.70-4.07%.

The newly developed method can quickly and accurately analyze MO and IMO. This simultaneous analysis is expected to study analyzing sugars.

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