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A Comprehensive Study on Triacylglycerols in Tobacco Leaves Using Liquid Chromatography and Atmospheric-Pressure Chemical-Ionization Mass Spectrometry*

by

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SUMMARY

Triacylglycerols in tobacco leaves were investigated to further understand their compositional and metabolic differences among various tobacco leaves and their changes during the curing processes. Non-aqueous reverse-phase chromatography for efficient separation of triacylglycerols and atmospheric-pressure chemical-ionization mass spectrometry for their detection were selected for quantitative analysis. For measuring their absolute amounts, authentic triacylglycerols comprised of the same fatty acids were introduced to generate calibration curves and to quantify the analytes.
A validation study using the combination of appropriate separation and authentic triacylglycerols produced very good results in terms of linearity (> 0.999), limit of detection (3–4 ng), limit of quantification (10–15 ng) and recovery ratio (80–120%). The quantification of triacylglycerols in tobacco leaves and a subsequent principal component analysis clarified for the first time their compositional and metabolic differences among various tobacco leaves. [Beitr. Tabakforsch. Int. 25 (2013) 627–637]

ZUSAMMENFASSUNG

Triglyzeride in Tabakblättern wurden untersucht, um die Unterschiede in ihrer Zusammensetzung und im Stoffwechsel sowie ihre Veränderungen während des Trocknungsprozesses in verschiedenen Tabakblättern besser zu verstehen. Für die quantitative Analyse wurden die nichtwässrige Umkehrphasenchromatographie zur effektiven Trennung sowie die Massenspektrometrie mittels chemischer Ionisation bei Atmosphärendruck zur Detektion der Triglyzeride eingesetzt. Zur Messung der absoluten Menge an Triglyzeriden wurden authentische Trigylzeride, die aus den gleichen Fettsäuren bestehen, eingeführt, mit deren Hilfe Kalibrationskurven erstellt und die Analyten quantifiziert wurden.
In einer Validierungsstudie, die mit einer Kombination aus geeigneter Trennung und authentischen Triglyzeriden durchgeführt wurde, wurden sehr gute Ergebnisse in den Bereichen Linearität (> 0.999), Nachweisgrenze (3–4 ng), Quantifizierungsgrenze (10–15 ng) und Wiederfindungsrate (80–120%) erzielt. Zum ersten Mal ist es somit gelungen, die Unterschiede bei Zusammensetzung und Stoffwechsel verschiedener Tabakblätter durch die Quantifizierung der in den Tabakblättern vorhandenen Triglyzeride sowie die anschließende Hauptkomponentenanalyse aufzuklären. [Beitr. Tabakforsch. Int. 25 (2013) 627–637]

RESUME

Les triacylglycérols dans les feuilles de tabac ont été examinés pour mieux comprendre les différences compositionnelles et métaboliques entre diverses feuilles de tabac et leurs modifications au cours des processus de fumage. La chromatographie en phase inverse non aqueuse pour la séparation efficace des triacylglycérols et la spectrométrie de masse par ionisation chimique à pression atmosphérique pour leur détection ont été retenues pour l'analyse quantitative. Pour mesurer leurs quantités absolues, des triacylglycérols authentiques composés des mêmes acides gras ont été introduits afin de générer des courbes d'étalonnage et de quantifier les analytes.
Une étude de validation utilisant la combinaison de séparation adéquate et des triacylglycérols authentiques a produit de très bons résultats en termes de linéarité

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(> 0,999), de limite de détection (3–4 ng), de limite de quantification (10–15 ng) et de taux de récupération (80–120%). La quantification des triacylglycérols dans les feuilles de tabac et une analyse subséquente des principaux composants ont permis de clarifier pour la première fois les différences compositionnelles et métaboliques entre diverses feuilles de tabac. [Beitr. Tabakforsch. Int. 25 (2013) 627–637]

KEYWORDS

Triacylglycerol; tobacco leaf (*Nicotiana tabacum*); NARPC; LC/APCI-MS; curing; positional isomer

ABBREVIATIONS

Triacylglycerol species are abbreviated using initials of fatty acids as follows: P, palmitic acid; S, stearic acid; O, oleic acid; L, linoleic acid; and Ln, linolenoic acid, e.g., glycerol esterified by palmitic acid, stearic acid and oleic acid is abbreviated to OPS.
Since positional isomers like sn-2 vs. sn-1 or sn-3 positions cannot be distinguished in the analysis of this research, the initials are alphabetically sorted like OPS. When special occasions that should refer to positional isomers are required, the initials have an asterisk to the right shoulder such as POS*. It stands for 1-palmitoyl-2-oleoyl-3-stearoyl-sn-glycerol, where oleic acid is not distributed in sn-1 or sn-3 position.

INTRODUCTION

Triacylglycerols are the major storage lipids in plant seeds, resins, flower petals and pollen grains, and are the energy sources for an enormous number of physiological processes. The universality and functionality are derived from the structural diversities of triacylglycerols which include various fatty acids ranging from saturated to unsaturated ones (1). Much attention has therefore been paid to studies to develop an analytical method for comprehensive triacylglycerols quantification and to compare the different composition among samples and to clarify each analyte's role in metabolism.
Tobacco leaves in various plants are already known for containing numerous components such as cembranoids, labdanoids, terpenoids, carotenoids, hydrocarbons, fatty acids, alkaloids, polyphenols, amino acids, sugars and organic acids. Many studies concerning these compounds have been executed in order to elucidate their differences in plant position, growing district, curing process for drying and senescence among various tobacco types (2–4). Some studies have focused on compositional changes during curing processes because they certainly determine quality, characteristics and type of tobacco leaf. However, except for the studies of fatty acids combining with glycerol in tobacco seed during germination and maturation (5, 6), little attention has been paid to the whole composition of triacylglycerols in tobacco leaves, the compositional differences of triacylglycerols among various tobacco leaves and their changes during curing processes. Clarification of these questions was considered crucially important to explain more about the qualities and types of tobacco leaves. For this reason, the objectives of this research are to develop a feasible analytical method for triacylglycerols in tobacco leaves, to investigate their compositional differences among several types of tobacco leaves and then to evaluate the relation of triacylglycerols with the types and curing processes of tobacco leaves.
Early analytical methods (5–7) for triacylglycerols began with their alkaline hydrolysis to identify the fatty acids combined with glycerol and the subsequent analysis of their methyl esters without clarifying the complete structures of the triacylglycerols. In more recent years, several approaches have been presented to determine the structures and compositions. Gas chromatography (GC) incorporating a high-temperature-tolerable capillary column coupled with a flame ionization detector (FID) or mass spectrometer (MS) has generally been accepted as a useful combination for analysis (8–10). Moreover, liquid chromatography (LC) using an octadecylsilane (ODS) column coupled with various detectors has become more popular for separating and identifying triacylglycerols. The reason for the frequent use of this combination is that non-aqueous reverse-phase chromatography (NARPC), a separation technique for reverse-phase liquid chromatography without water, was expected to efficiently separate low polarity components (11). For this reason, the following detectors have been coupled with the separation system for triacylglycerols: refractive index detector (RID) (12), evaporative light scattering detector (ELSD) (13–15), atmospheric pressure chemical ionization mass spectrometer (APCI-MS) (10, 16–22) and atmospheric pressure photoionization mass spectrometer (APPI-MS) (23).
Above all, it should be noted that APCI-MS is a highly effective instrument for the identification of triacylglycerols. When a triacylglycerol molecule is introduced into the APCI-MS, it is protonated and subsequently decomposed into three types of diacylglycerol ions through the loss of one of its three fatty acids. This ionization process makes it possible to determine the structure of the triacylglycerol (17, 18, 24). Moreover, this latest separation technique as well as detection has been further improved. A silver ion chromatography column is known to separate the positional isomers of triacylglycerols (10, 25, 26). The combination of the silver ion column with another separation system, that is, two-dimensional chromatography, has accomplished the separation of positional isomers of triacylglycerols (27, 28). Supercritical carbon dioxide is also an effective eluent to separate triacylglycerols (29).
These techniques were thought to be applicable to the analysis of tobacco leaves. Principally, NARPC with APCI-MS was considered to be a powerful combination to reveal the actual structures and compositions of triacylglycerols in tobacco leaves. However, even if this combination was employed, it would have provided us only with qualitative results owing to the difficulty in preparing authentic samples of all of the corresponding triacylglycerols. In order to determine the quantitative amounts of triacylglycerols and statistically evaluate the results, the idea of response factor (21) was introduced.

This report first examines the applicability of LC/APCI-MS for separation triacylglycerols, then validates the analytical procedure for tobacco leaves and finally analyzes the results acquired from several types of tobacco leaves and curing processes.

MATERIALS AND METHODS

Materials

Tobacco leaves, *Nicotiana tabacum* (Flue-cured Virginia (FCV), Burley (BLY), Oriental (ORI), Dark-air cured (DAC), Dark-fire cured (DFC), Sun-air cured (SAC) and stems) and *Nicotiana rustica* were stored in a warehouse of Japan Tobacco, Inc. and were used as samples for the analysis of triacylglycerols. Uncured tobacco leaves (AC0) were harvested in the usual manner from an in-house plantation of Japan Tobacco, Inc. These uncured samples were stored in a room with constant temperature (22 °C) and humidity (50%) for drying comparable to the air-curing process and then they were also used for analysis. Solvents for extraction and liquid chromatography were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). All solvents used were HPLC grade. Authentic standards of triacylglycerols for identification and quantification were purchased from the following sources: Tripalmitin, CAS# 555-44-2 (PPP) and tristearin, CAS# 68334-00-9 (SSS) were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan); triolein, CAS# 122-32-7 (OOO); trilinolein, CAS# 537-40-6 (LLL) and trilinolenin, CAS# 14465-68 (LnLnLn) were purchased from Sigma-Aldrich (St. Louis, MO, USA); (±)-1,2-dipalmitoyl-3-oleoylglycerol, CAS# 37179-82-1 (PPO*); (±)-1,2-distearoyl-3-oleoylglycerol, CAS# 51195-71-2 (SSO*); (±)-1-palmitoyl-2-oleoyl-3-stearoylglycerol, CAS# 2190-27-4 (POS*) and (±)-1-stearoyl-2-palmitoyl-3-oleoylglycerol, CAS# 59891-29-1 (SPO*) were purchased from Indofine Chemical Co., Inc. (Hillsborough, NJ, USA).

Sample preparation

Approximately 100 g of tobacco leaves was pulverized using an M-2 Mill (Nara Machinery Co., Ltd., Tokyo, Japan) to pass through a 1 mm mesh. The pulverized samples were subsequently assigned to an extraction apparatus ASE200 (Dionex Corporation, Sunnyvale, CA, USA). 2.5 g of the pulverized sample was transferred into a pressure-resistant vessel that was then filled with sea sand to eliminate all air gaps. Extraction was done using *n*-hexane for all the samples. The *n*-hexane extraction was performed at 70 °C and 2000 psi. The extracts from 10 g of tobacco leaves (four vessels) were combined into a 250 mL volumetric flask. The extract of 10 mL was evaporated *in vacuo,* dissolved using acetone and readjusted to the original volume of 10 mL. This acetone solution was filtered using a Millex-LG with a 0.20 µm pore size (Millipore Corporation, Bedford, MA, USA) to remove insoluble components. The filtered acetone solution was transferred into a tightly sealed, amber glass vial under darkness (Agilent Tech., Santa Clara, CA, USA) and an aliquot of 50 µL was subsequently injected into the analytical instrument.

Uncured tobacco leaves were treated differently from cured-tobacco leaves due to the greater amount of water. The uncured leaves, which were weighed and soaked in acetone solution in advance, were homogenized with an OMNI GLH homogenizer (Omni International, Inc., Warrenton, VA, USA) configured at 4000 rpm. The samples were carefully filtered with filter paper #1 (Advantec Toyo, Inc., Tokyo, Japan) and then the filtrate was combined into a 250 mL volumetric flask using acetone. The subsequent procedure was the same used with cured tobacco leaves as mentioned earlier.

Moisture content measurement

Approximately 2 g of pulverized tobacco leaves was weighed and dried at 80 °C for 3 hours. After drying, the sample was cooled in a desiccator for an hour and then weighed. The reduction in weight was assumed to be moisture that was later used to evaluate the amount of triacylglycerols on a dry weight basis (DB). The moisture content of uncured and partially-cured tobacco leaves was determined from the analysis of water in their acetone solutions with an Agilent 6980 GC (Agilent Tech., Santa Clara, CA, USA) system incorporating a thermal conductivity detector (TCD).

Instrument and column conditions

High-Performance Liquid Chromatography (HPLC) analysis was performed using an Agilent 1200 HPLC system consisting of the following components: G1379B degasser; G1312B Binary pump SL; G1367C Hip-ALS SL; G1330B FC/ALS therm; and 6130 quadrupole APCI-MS (Agilent Tech., Santa Clara, CA, USA). An Excelpak SIL-C18/5C column (250 mm × 4.6 mm I.D., 5 µm, Yokogawa Analytical Systems, Tokyo, Japan (currently available from Agilent Tech.)) was used under the following conditions: Mobile phase A was acetonitrile; mobile phase B was acetone; flow rate was 1.0 mL/min; gradient conditions for A were 100% at 0 min, 30% at 10 min, 20% at 30 min, 0% at 40 min, and 0% (i.e., B: 100%) until 55 min; injection volume was 50 µL; and the column temperature was 25 °C. The APCI-MS was used under the following conditions: capillary voltage 4000 V; corona current 10 µA; drying gas flow 5 mL/min; drying gas temperature 350 °C, fragmentor voltage 200 V, nebulizer pressure 60 psi; vaporizer temperature 500 °C; signal measurement period 15–55 min. The APCI-MS was also used for quantification in selected ion monitoring (SIM) mode configured at the following m/z: 551.7, 573.7, 575.7, 577.7, 579.7, 595.7, 597.7, 599.7, 601.7, 603.7, 605.7 and 607.7.

Statistical analysis of data

All data are presented as the mean with standard deviation. The quantification results of triacylglycerols were subjected to principal component analysis (PCA) using JUSE-statworks/V4.0 sw4 (Union of Japanese Scientists and Engineers, Tokyo, Japan).

RESULTS AND DISCUSSION

Separation and identification of triacylglycerols in tobacco leaves

This research began with the separation and identification of triacylglycerols in tobacco leaves. Because the separation of the other low polarity components in tobacco leaves by NARPC had already been examined (30, 31), the same protocol was first applied to the analysis of triacylglycerols. APCI-MS was selected as the instrument to determine which types of fatty acids esterify glycerol and to quantify the analytes as in previous reports (17, 18, 24). Moreover, because the report concerning hydrolyzed crude lipids in tobacco leaves revealed that the fatty acids of triacylglycerols were mainly linolenic (Ln), linoleic (L), oleic (O), palmitic (P) and stearic (S) (32, 33), targets were focused on the triacylglycerols consisting of these five fatty acids. For this reason, SIM mode was initially configured at the m/z of twelve diacylglycerol ions as detailed earlier. Triacylglycerols in Oriental leaf (ORI) harvested in Turkey and giving specific taste and aroma to cigarette are shown in the extracted ion chromatograms (EIC) in SIM mode (Figure 1). Although some peaks (e.g., LnOS and LOP at 30 min of retention time on EIC of 602 m/z) are not fully resolved, all the other peaks showed sufficient separation

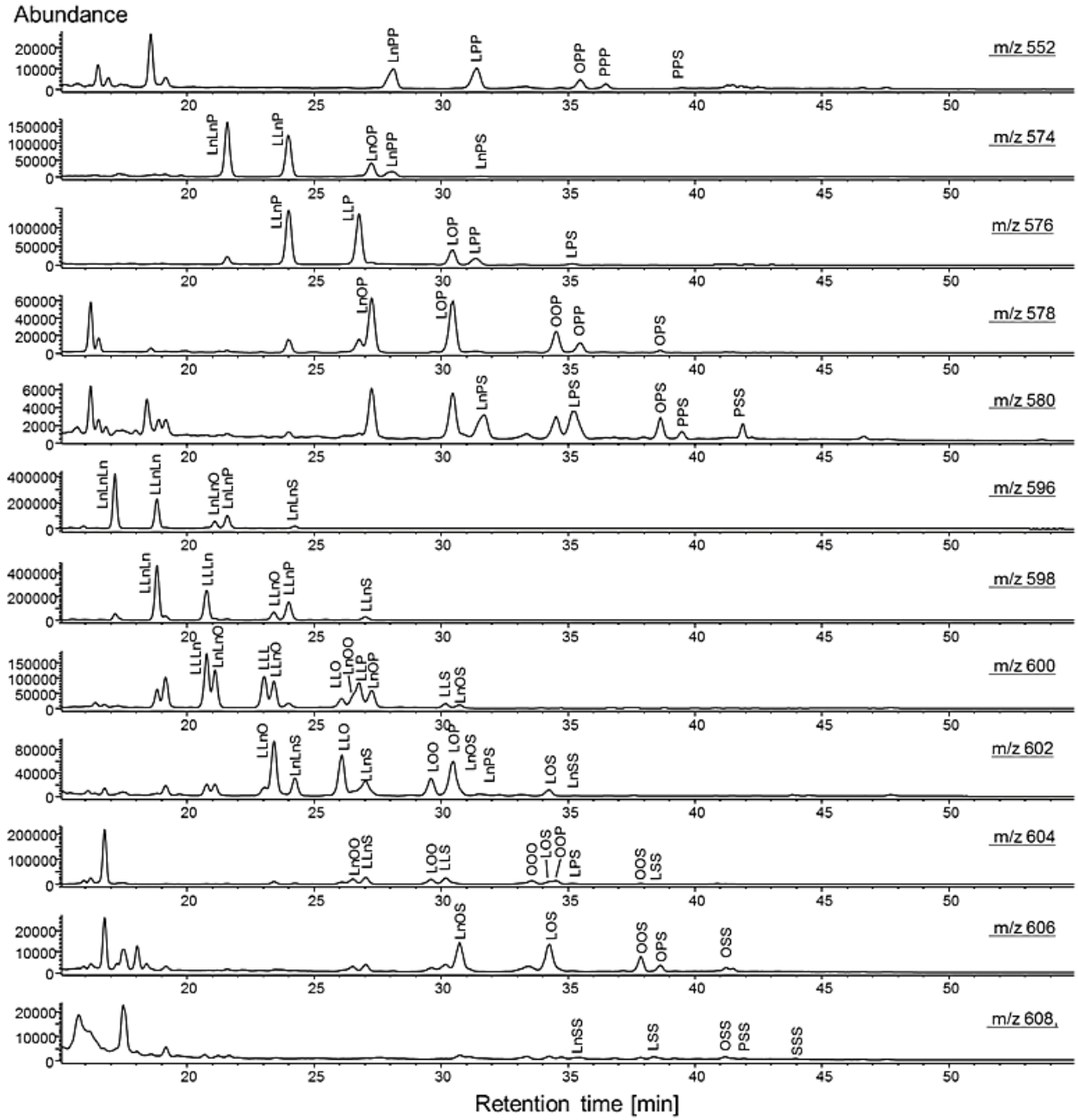

Figure 1. EIC showing peaks of triacylglycerols in ORI. The analytical method is summarized in Instrument and column condition. The abundances on extracted ion chromatograms (EIC) are normalized due to a different scale. Abbreviations of the fatty acids are as follows; L: linoleic acid, Ln: linolenoic acid, O: oleic acid, P: palmitic acid, and S: stearic acid.

Table 1. Slope of calibration curve, determination coefficient, LOD and LOQ measured by HPLC/APCI-MS in SIM mode. Range of calibration curve was configured from 10 to 6000 ng/mL. LOD and LOQ were measured 10 times.

Compound	Slope of calibration curve (mL/ng)	Determination coefficient R^2	Actual conc. (ng/mL)	Detected conc. (ng/mL)	±SD (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	RSD (%)
LnLnLn	1.55 E+03	0.9999	12.6	13.2	1.1	3.4	11.3	8.6
LLL	2.14 E+03	0.9999	12.5	13.1	1.3	3.8	12.7	9.7
OOO	3.29 E+03	1	12.0	13.0	1.4	4.2	14.0	10.8
PPP	2.33 E+03	1	12.0	13.5	1.0	3.0	10.2	7.5
SSS	3.16 E+03	1	14.1	14.4	1.6	4.9	16.3	11.3

Abbreviations of the fatty acids are as follows; L: linoleic acid, Ln: linolenoic acid, O: oleic acid, P: palmitic acid, and S: stearic acid.

for identification and quantification in an analysis time of 50 min. Each triacylglycerol was identified by the peaks of diacylglycerol ions at the same retention times and their elution orders are dependent on the total number of carbons and double bonds as previously reported (17, 19, 20). This procedure enabled us to identify 35 triacylglycerols in tobacco leaves.

Validation study with authentic standard triacylglycerols

Slopes of calibration curves and determination coefficients of authentic standard triacylglycerols (LnLnLn, LLL, OOO, PPP and SSS) are summarized in Table 1. The calibration curves drawn with areas of diacylglycerol ion peaks derived from triacylglycerols gave excellent linearity in excess of 0.999 in the range from 10 to 6000 ng/mL. However, the slopes showed slight differences among triacylglycerols, e.g., LnLnLn gave the lowest figure. This suggests that the leaving-group ability of the fatty acid from the triacylglycerol has a slight effect on the formation of diacylglycerol ions. Therefore, a standard triacylglycerol response factor could not simply be applied to quantify another triacylglycerol. Nevertheless, there have been several attempts to address this issue. Some analysts state that the different responses among triacylglycerols can be converted into the proportional values to achieve more accurate results (21). In the current study, the author thereby applied the same strategy; the peak intensities of standard triacylglycerols including the same fatty acids were averaged depending on the composition of fatty acids of analyte (e.g., LLnO was quantified with the averaged calibration curves of LLL, LnLnLn and OOO). While the verification test for this procedure is provided later, the limit of detection (LOD) and limit of quantification (LOQ) measured for the five authentic standard triacylglycerols are summarized in Table 1. Both LOD and LOQ gave acceptable results for quantifying triacylglycerols in tobacco leaves. The results at the low concentrations quantified by authentic triacylglycerols did not deviate significantly from the actual amounts.

Validation study on quantification procedure

Table 2 shows the quantification results of several authentic triacylglycerols (POS*, SPO*, SSO* and PPO*) using their diacylglycerol ions (OP^+, PS^+, OS^+, PP^+ and SS^+) and the calibration curves by authentic triacylglycerols (LnLnLn, LLL, OOO, PPP and SSS). The amounts of POS*(OP^+) quantified by calibration curves of LnLnLn (13.29 μg/mL) and LLL (9.65 μg/mL) were much higher than those by OOO (6.30 μg/mL), PPP (8.92 μg/mL), SSS (6.57 μg/mL) and the actual amount (6.09 μg/mL). The analytes which did not include the same fatty acids as the standard components were unlikely to be quantified as an equivalent amount. Similar tendencies were observed in the other triacylglycerols (SPO*, SSO* and PPO*). The leaving-group ability of the fatty acid was therefore considered a principal factor on the peak intensity of the diacylglycerol ion. Consequently, a triacylglycerol should be at least quantified by another triacylglycerol including the same fatty acid as the analyte.

Additionally, the quantification results indicated another effect than the leaving-group ability. For example, POS* (OP^+) quantified by a calibration curve of SSS (SS^+) gave 6.57 μg/mL. On the other hand, POS*(OS^+) by PPP (PP^+) gave 9.65 μg/mL and POS* (PS^+) by OOO (OO^+) was 4.89 μg/mL. The results did not agree with each other or the actual amount (6.09 μg/mL), even though both of the fatty acids from the standard component and analyte were the same in all cases. Above all, the quantification result using the diacylglycerol ion from the sn-2 position (e.g., SO^+ of SPO*) seemed to show the smallest result. This tendency is consistent with previous reports (17, 19, 20, 34). According to these references, the elimination from sn-1 or sn-3 positions is expected to result in a six-member transition state in the mass analyzer. Meanwhile, elimination from the sn-2 position presumably gives a five-member transition state that may be thermodynamically less stable than a six-member state (34). In other words, leaving-group ability in sn-1 or sn-3 positions is higher than that in sn-2 position. After all, because the authentic standards like OOO give the same diacylglycerol ions derived from both sn-1 or sn-3 and sn-2, it is quite difficult to achieve a quantification method for triacylglycerols even accounting for positional isomers. Therefore, in this study, the author averaged all the results acquired from the standard components including the same fatty acids as the analyte.

Quantification steps for the triacylglycerols were done as follows. Calibration curves for quantifying triacylglycerols in tobacco leaves were initially drawn by peak areas of diacylglycerol ions from authentic standard triacyl-glycerols (LnLnLn, LLL, OOO, PPP and SSS). The slopes of the calibration curves were divided by three because the intensities of these standard triacylglycerols were tripled by elimination of three fatty acids. The calibration curve was

Table 2. Quantification results of authentic triacylglycerols (POS*, SPO*, SSO* and PPO*) using their corresponding diacylglycerol ion (OP+, OS+, PS+, PP+ and SS+) and the calibration curves of authentic triacylglycerols (LnLnLn, LLL, OOO, PPP and SSS). Measurements were replicated ten times. The bold letter shows that eliminated fatty acids from authentic triacylglycerols for calibration and quantification are the same.

Authentic triacylglycerol	Diacylglycerol ion for quant.	Actual conc. (μg/mL)	Authentic triacylglycerol for quant.	Calculated conc. (μg/mL)	SD (μg/mL)	RSD (%)	Ratio (%)	Average conc. (μg/mL)	Ratio (%)
POS*	OP^+	6.09	LnLnLn	13.29	±0.99	7.4	218	7.04	116
			LLL	9.65	±0.64	6.6	158		
			OOO	6.30	±0.44	7.0	103		
			PPP	8.92	±0.67	7.5	146		
			SSS	**6.57**	**±0.48**	**7.3**	**108**		
	OS^+	6.09	LnLnLn	14.38	±0.93	6.5	236		
			LLL	10.44	±0.65	6.2	172		
			OOO	6.82	±0.39	5.7	112		
			PPP	**9.65**	**±0.64**	**6.6**	**159**		
			SSS	7.12	±0.51	7.2	117		
	PS^+	6.09	LnLnLn	10.30	±0.55	5.3	169		
			LLL	7.48	±0.37	4.9	123		
			OOO	**4.89**	**±0.31**	**6.3**	**80**		
			PPP	6.92	±0.51	7.4	114		
			SSS	5.10	±0.39	7.6	84		
SPO*	OP^+	4.51	LnLnLn	9.42	±1.05	11.1	209	4.74	105
			LLL	6.83	±0.61	8.9	151		
			OOO	4.46	±0.42	9.3	99		
			PPP	6.31	±0.61	9.6	140		
			SSS	**4.64**	**±0.26**	**5.6**	**103**		
	OS^+	4.51	LnLnLn	6.53	±0.58	8.8	145		
			LLL	4.74	±0.35	7.4	105		
			OOO	3.09	±0.20	6.6	69		
			PPP	**4.37**	**±0.29**	**6.6**	**97**		
			SSS	3.22	±0.18	5.5	71		
	PS^+	4.51	LnLnLn	10.99	±1.12	10.2	244		
			LLL	7.97	±0.67	8.4	177		
			OOO	**5.20**	**±0.42**	**8.0**	**115**		
			PPP	7.37	±0.63	8.6	163		
			SSS	5.42	±0.28	5.1	120		
PPO*	OP^+	5.38	LnLnLn	9.93	±0.92	9.3	185	6.31	117
			LLL	7.21	±0.58	8.1	134		
			OOO	4.72	±0.61	13.0	88		
			PPP	6.70	±0.95	14.2	124		
			SSS	**4.93**	**±0.60**	**12.1**	**92**		
	PP^+	5.38	LnLnLn	12.46	±0.96	7.7	232		
			LLL	9.04	±0.52	5.8	168		
			OOO	**5.92**	**±0.57**	**9.7**	**110**		
			PPP	8.38	±0.89	10.6	156		
			SSS	6.17	±0.52	8.5	115		
SSO*	OS^+	5.80	LnLnLn	11.00	±0.85	7.7	190	6.66	115
			LLL	7.98	±0.48	6.0	138		
			OOO	5.21	±0.31	5.9	90		
			PPP	7.37	±0.45	6.1	127		
			SSS	**5.43**	**±0.27**	**5.0**	**94**		
	SS^+	5.80	LnLnLn	16.66	±1.47	8.8	287		
			LLL	12.08	±0.77	6.4	208		
			OOO	**7.89**	**±0.60**	**7.6**	**136**		
			PPP	11.17	±0.89	7.9	192		
			SSS	8.22	±0.41	4.9	142		

Abbreviations of the fatty acids are as follows; L: linoleic acid, Ln: linolenoic acid, O: oleic acid, P: palmitic acid, and S: stearic acid.

Table 3. Recovery ratio of authentic standard triacylglycerols added to actual tobacco extract from ORI harvested in Turkey.

Compound	Added (ng/mL)	Detected (ng/mL)	±SD (ng/mL)	Recovery (%)	RSD (%)
LnLnLn	0	2655.6	77.9	—	2.9
	1603.7	4297.2	148.3	102.4	3.5
	3207.4	6072.6	506.4	106.5	8.3
	5078.4	7644.6	213.1	98.2	2.8
LLL	0	159.7	5.7	—	3.6
	46.8	215.1	11.5	118.3	5.3
	140.5	325.9	12.7	118.3	3.9
	333.7	531.5	20.5	111.4	3.8
OOO	0	53.2	3	—	5.6
	28.4	84.8	3.6	111	4.2
	45	104.5	5.5	113.9	5.3
	135	203.7	8.2	111.5	4
PPP	0	23.6	0.7	—	3.1
	9.5	35.2	4.2	121.1	11.8
	28.5	55.4	1.1	111.1	2
	45.2	72	4.3	107.1	5.9
SSS	0	2.5	1.4	—	57.3
	2.4	3.9	0.4	62.2	9.7
	7.1	9	0.9	92.4	10.5
	11.2	13.2	1.6	95.9	12.5

Abbreviations of fatty acids are as follows; L: linoleic acid, Ln: linolenoic acid, O: oleic acid, P: palmitic acid, and S: stearic acid.

then used to quantify the diacylglycerol ions of analytes with elimination of the same fatty acid as the authentic triacylglycerols, e.g., LLO (LL^+), LOO (LO^+) and PPO (PP^+) were quantified by OOO (OO^+). In the case of SSP quantification, the diacylglycerol ion SP^+ was considered to be doubled due to elimination of two stearic acids from sn-1 or sn-3 and sn-2 positions of the glycerol molecule. The area of SP^+ was therefore divided by two, and then quantified by SSS. On the other hand, SSP (SS^+) was quantified by the divided PPP without division of the area of SSP. These results were in the end averaged in order to offset the effect of positional isomers. The quantification results in Table 2 by this method appear closer to the actual amounts.

Recovery ratio

Recovery ratios that were measured through addition of authentic standard triacylglycerols to the extract of ORI are summarized in Table 3. Almost all the concentrations provided acceptable results ranging from 80 to 120%. The lowest concentration of SSS gave an unfavorable standard deviation due to its proximity to its LOQ; all the other results from identifiable levels gave acceptable standard deviations for quantification.

Triacylglycerols in various tobacco leaves

Some quantification results of triacylglycerols in tobacco leaves are summarized in Table 4. The most distinguishable feature in the table was the difference between FCV and BLY concentrations. The total amount of triacylglycerols in FCV was higher than BLY. FCV also gave a higher proportion of triacylglycerols made up of unsaturated fatty acids such as LLLn, LnLnL, LnLnLn, etc. In contrast, BLY gave a relatively higher proportion of triacylglycerols consisting of saturated fatty acids such as OPP, OPS, OOS, etc.

In previous reports, MATSUZAKI *et al.* confirmed a more rapid decrease of glycolipid- and phospholipid-containing unsaturated fatty acids than saturated fatty acids during the curing process (32, 33). Because glycolipids and phospholipids are usually related to triacylglycerols in metabolic pathways, triacylglycerols were expected to show a similar tendency. Moreover, FCV is dried at a high temperature (about up to 70 °C) during the early stage of the curing process (2), and BLY is conversely dried at an ambient temperature for a longer drying time than FCV. Taking these factors into consideration, the observation that FCV has the higher amount and greatest proportion of unsaturated fatty acids seems reasonable.

ORI is normally dried under sunlight, and then cured in a barn for further fermentation. Its results show a slightly higher proportion of triacylglycerols including Ln than FCV, while the total amount seems equivalent to FCV. DFC, a smoke-cured leaf, shows a triacylglycerol composition that is mainly oleic acid. DAC, which was processed under ambient temperature, gave almost the same amount and composition of triacylglycerols as BLY. Tobacco leaves with similar curing processes did not show substantial difference in the amount and composition of triacylglycerols. Tobaccos from different growing districts, but cured similarly, have a similar composition of triacylglycerols. Overall, tobacco leaves appear to be classified into two types: flue-cured (FCV and ORI) and air-cured type (BLY and DAC).

Quantification of triacylglycerols was expanded to other tobacco leaves to figure out the overall tendency. To this end, principal component analysis (PCA) was introduced to consolidate quantification results and to find out crucial factors to classify tobacco leaves. The starting matrixes of data that are subjected to PCA are basically two types: Variance-covariance matrix (VCM) and correlation coefficient matrix (CCM). VCM can include the quantitative meaning to the result of PCA, but it is not applicable to data consisting of different units. CCM is more generally used because normalized data with different units are applicable to the PCA. In this report, the author selected VCM because the quantification results of triacylglycerols were approximated to actual amounts by a response factor.

Data from 60 tobacco leaf samples were subjected to PCA. Score and factor loading plots are shown in Figure 2. The score plot (upper in Figure 2) gives a cumulative contribution ratio of over 80%, which is considered sufficiently reliable. The primary ingredient clearly distinguished FCV and ORI from BLY and DAC. Because many components on the factor-loading plot (lower in Figure 2) were preferably placed on the right side, the primary ingredient is therefore interpreted to be the total amount of triacylglycerols. The same tendency is observed in the case of tobacco stems. The stems of FCV and BLY are clearly separated in the same direction as the primary ingredient.

Table 4. Quantification results for triacylglycerols in tobacco leaves. Measurements were replicated six times.

Sample	FCV (BRA)			FCV (TZA)			BLY (USA)			BLY (BRA)			ORI (TUR)			ORI (GRC)			DFC (TZA)			DAC (PHL)		
Compound	Avg	SD	RSD	Avg	SD	RSD	Avg	SD	RSD	Avg	SD	RSD	Avg	SD	RSD	Avg	SD	RSD	Avg	SD	RSD	Avg	SD	RSD
	(μg/mL)		(%)	(μg/mL)		(%)	(μg/mL)		(%)	(μg/mL)		(%)	(μg/mL)		(%)	μ(g/mL)		(%)	(μg/mL)		(%)	(μg/mL)		(%)
LnLnLn	70.1	±4.5	6.4	82.4	±21.2	25.7	28.4	±6.4	22.5	14.9	±4.4	29.1	122	±7.5	6.2	64.2	±5.0	7.9	7.5	±2.2	28.5	1.5	±0.2	13
LLnLn	121	±32.4	26.7	87.5	±5.2	5.9	29.1	±10.4	35.8	13.3	±3.0	22.6	93.5	±5.8	6.2	50.4	±2.5	4.9	10.5	±0.9	8.8	1.8	±0.2	9.6
LLLn	85.2	±4.4	5.2	55.8	±7.0	12.6	16.8	±7.5	44.5	8.5	±2.0	23.9	36.9	±4.2	11.4	21.9	±2.0	9	11.3	±2.3	20.8	1.5	±0.3	22.5
LnLnO	29.2	±2.3	7.9	12.1	±1.0	8.4	6.1	±3.0	48.4	2.8	±0.8	27.6	35	±2.3	6.5	15.9	±1.0	6	1.6	±0.4	25.7	0.9	±0.2	19.7
LnLnP	50.9	±3.4	6.7	58.8	±3.9	6.7	24.9	±9.2	37.1	15	±3.5	23.5	109	±10.8	9.9	57.5	±3.9	6.8	9.7	±0.8	8.4	2.6	±0.3	13.4
LLL	18	±1.4	7.5	9.1	±0.8	8.8	5.7	±2.0	35	6.4	±0.6	9.0	9.3	±1.4	15.5	22.5	±4.4	19.4	6.6	±0.5	8.2	0.8	±0.1	15.5
LLnO	43.7	±3.3	7.6	15.1	±0.9	5.9	9.1	±2.1	22.8	5.1	±0.9	17.0	29.5	±2.5	8.6	16.4	±1.4	8.3	5	±0.5	9.3	1.6	±0.2	11.5
LLnP	52.6	±5.7	10.8	42.8	±2.6	6.2	21	±5.2	24.8	9.9	±2.6	26.6	48.9	±5.8	11.9	28.2	±2.0	7.3	21.6	±3.7	17.3	2.3	±0.2	10.1
LnLnS	9.6	±1.1	11.2	11	±0.9	8	4	±0.5	12.3	1.9	±0.5	25.2	24.6	±3.1	12.4	11.8	±1.2	10	1.4	±0.4	27.7	0.3	±0.1	15.7
LLO	16.5	±1.5	9.1	6	±0.7	12	7.8	±3.6	46.6	8.7	±2.9	33.4	8.6	±0.5	6	12.8	±1.4	10.9	16.4	±2.2	13.5	2.4	±0.2	9
LnOO	13.3	±1.9	14	4	±0.8	20.4	2.9	±1.1	38.5	4.9	±3.2	65.2	10.1	±1.2	11.5	6.1	±0.4	6.3	3.9	±0.7	16.7	2.3	±1.0	43.5
LLP	45.2	±12.3	27.2	26.4	±3.3	12.6	13.5	±5.1	37.7	10.1	±2.1	20.5	21.6	±4.0	18.7	21.8	±1.6	7.4	21.9	±1.5	6.9	2.7	±0.7	24.9
LLnS	20.2	±2.7	13.3	17.7	±1.9	11	4.8	±1.6	32.5	3.3	±0.6	19.3	19.5	±2.9	14.9	10.4	±0.8	7.8	3.9	±0.3	8.4	0.9	±0.1	14.1
LnOP	34.5	±1.8	5.3	18.3	±4.0	22.1	8.3	±2.9	34.9	4.6	±1.1	24.0	43.4	±5.7	13.1	21.1	±1.2	5.9	6.3	±0.4	6.6	2.2	±0.3	13
LnPP	8.7	±0.7	8.3	10.6	±1.6	15.4	3.2	±1.1	33.5	2	±0.5	23.9	17.9	±2.1	12	9.4	±0.6	6.1	2.7	±0.9	34.7	0.7	±0.2	24.6
LOO	10.2	±0.7	6.6	3.3	±0.3	9.1	8.7	±4.1	46.4	6.6	±0.8	12.7	5.8	±0.5	9.4	6.1	±0.4	6.5	34.3	±2.3	6.7	2.3	±0.2	6.7
LLS	8.2	±0.6	7.3	5.3	±0.4	7.4	3.2	±1.2	36.5	2.4	±1.0	39.2	5.4	±0.5	8.8	5.4	±0.5	10	8.6	±0.4	4.8	0.8	±0.1	7.3
LOP	56.3	±3.4	6	39.2	±2.0	5.2	22.9	±6.3	27.5	12.2	±3.7	30.6	48.5	±5.1	10.6	28.8	±1.7	6	48.9	±4.8	9.9	4	±0.4	10.6
LnOS	6.2	±0.3	5.6	3.2	±0.3	10.4	1.2	±0.4	33.1	0.9	±0.3	32.5	8.5	±0.7	8.2	3.5	±0.2	5.8	1.7	±0.2	9.3	0.6	±0.0	3.6
LPP	7.2	±0.6	8.2	8.5	±1.1	12.6	11.7	±4.3	36.6	3.9	±1.0	25.1	7.8	±0.7	8.9	5.6	±0.8	14.1	40.3	±3.4	8.4	3	±0.2	7.1
LnPS	2.7	±0.2	8	3.1	±0.2	7.7	1	±0.4	35.8	0.7	±0.2	27.5	7.3	±0.7	9.5	3.2	±0.6	17.4	1.3	±0.3	22.5	0.3	±0.1	38.9
OOO	3.1	±0.3	9.3	2	±0.4	19.1	6.3	±2.9	47	4.5	±1.1	25.3	2.3	±0.3	14.5	2	±0.1	2.9	27.9	±3.2	11.4	1.1	±0.0	1.7
LOS	15.6	±0.6	3.8	7.4	±0.6	7.7	11.6	±3.6	31.4	5.7	±2.0	35.4	10.3	±1.0	10.1	7.6	±0.4	5.6	45.2	±2.8	6.3	2.8	±0.2	5.5
OOP	7	±1.0	13.7	5.2	±0.9	16.4	15.8	±6.4	40.6	6.5	±1.9	29.7	5.1	±0.6	11.1	3.7	±0.5	13.1	91.1	±13.4	14.7	3.8	±0.5	12.2
LPS	2.2	±0.3	12.1	3.1	±0.6	17.8	8.2	±3.1	38.3	2	±0.5	24.5	3	±0.2	6.3	2.1	±0.3	14	23.5	±2.6	11.2	1.4	±0.3	20
LnSS	0.6	±0.1	9.6	1	±0.2	16.8	0.4	±0.3	67.3	0.2	±0.1	32.8	1.6	±0.3	17.4	0.8	±0.1	17.9	0.4	±0.1	33.1	0.3	±0.1	51.7
OPP	2.8	±0.3	12.1	6.4	±0.9	14.2	19.8	±8.1	40.7	4.1	±1.1	27.4	3.8	±0.5	13.9	2.6	±0.3	12.4	91	±10.0	11	4.8	±0.9	18.8
PPP	0.5	±0.1	21	0.6	±0.1	17.2	0.4	±0.1	36.1	0.2	±0.1	42.9	0.9	±0.1	12.8	0.6	±0.1	10.3	0.7	±0.1	15.2	0.1	±0.1	107
OOS	1.3	±0.1	8.9	1.2	±0.2	15.8	6.2	±2.3	37.7	2	±0.5	26.6	1.1	±0.2	13.8	0.7	±0.1	14.1	30.6	±3.1	10.3	1	±0.4	38.7
LSS	0.5	±0.0	7.6	0.6	±0.2	28.4	1.8	±0.7	39.8	0.5	±0.2	29.2	0.7	±0.0	6.2	0.6	±0.1	22.4	5	±0.8	16.7	0.4	±0.1	34.1
OPS	1.2	±0.1	11.2	3	±0.4	11.8	15.6	±6.2	39.9	2.9	±1.2	39.7	1.7	±0.2	11.7	1.1	±0.1	12.2	61.8	±6.9	11.1	2.7	±0.3	10.2
PPS	0.2	±0.0	20	0.3	±0.0	15.1	0.3	±0.1	38.6	0.2	±0.1	40.0	0.5	±0.0	7.2	0.4	±0.1	17.3	0.6	±0.1	14.2	0.4	±0.7	158.6
OSS	0.4	±0.1	24.5	0.9	±0.1	11.9	2.8	±1.9	66.4	0.5	±0.3	57.2	0.6	±0.1	16.3	0.5	±0.1	15.6	12.4	±1.0	8.2	0.6	±0.2	38.3
PSS	0.2	±0.0	26.9	0.2	±0.1	25.4	0.1	±0.1	79.5	0.5	±0.7	144.1	0.7	±0.1	9.3	0.3	±0.1	19.4	0.3	±0.0	12.1	0.1	±0.0	26.6
SSS	0	±0.0	164	0.1	±0.1	117	0.1	±0.1	119	0.2	±0.3	123.7	0.1	±0.0	37.3	0.1	±0.0	36.5	0.1	±0.0	44.6	0.1	±0.0	13.2
Total	745			552			323.8			168.0			745.6			446.2			656			55		

Abbreviations of the tobacco leaves, growing district, and fatty acids are as follows; FCV: Flue-cured Virginia, BLY: Burley, ORI: Oriental, DAC: Dark-air cured, DFC: Dark-fire cured, BRA: Brazil, TZA: Tanzania, USA: The United States of America, TUR: Turkey, GRC: Greece, PHL: Philippines, L: linoleic acid, Ln: linolenoic acid, O: oleic acid, P: palmitic acid, and S: stearic acid.

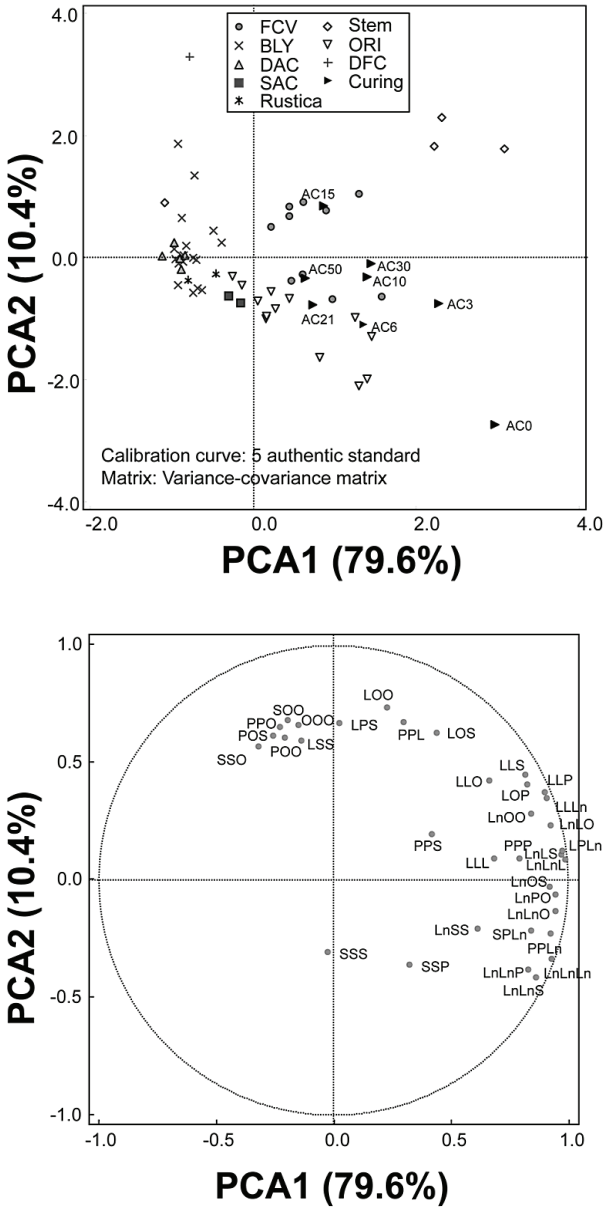

Figure 2. Score plot (upper) and loading plot (lower) given by PCA using quantification results of triacylglycerols in tobacco leaves. FCV during curing process were plotted by the eigenvector calculated from principal component analysis (PCA) and applied to cured tobacco leaves. Abbreviations of the tobacco leaves and fatty acids are as follows; FCV: Flue-cured Virginia, BLY: Burley, ORI: Oriental, DAC: Dark-air cured, DFC: Dark-fire cured, L: linoleic acid, Ln: linolenoic acid, O: oleic acid, P: palmitic acid, and S: stearic acid.

In other words, the compositional differences between flue-cured and air-cured types are similarly observed even in their stems. In addition, while the stems of FCV included a greater amount of triacylglycerols than the leaves of FCV (data not shown), the stems of FCV on the score plot are placed on the right of the leaves of FCV. This result also supported the interpretation of PCA shown above.

There is another specific tendency on the score plot. FCV and ORI are clearly separated in the direction of a secondary ingredient. Because the components mainly consisting of Ln on the loading plot are placed on the lower right, they are considered as the main factors to distinguish FCV from ORI. Moreover, DFC is completely isolated from the other tobacco leaves. Because the components mainly consisting of O are placed at the upper left, they are also considered to be the principal reason to distinguish DFC and other tobacco leaves. The secondary ingredient is consequently thought to distinguish FCV from ORI and characterize DFC. Totally, the composition of triacyl-glycerols is quite effective to elucidate the types of various tobacco leaves without regard to plant positions and growing districts.

Figure 2 additionally shows the time-dependent change of FCV samples stored in the constant temperature and humidity room. The principal component scores of the FCV samples were achieved from substitution of the respective quantitative data to the eigenvector estimated by PCA for cured-tobacco leaves. Because uncured-tobacco leaf (AC0) contains the greatest amount of triacylglyerols, mainly consisting of Ln, it was placed on the lower right of the score plot. As the samples were dried, their positions gradually approached the area between FCV and BLY. Although a series of AC samples did not reach the positions of BLY even after curing, the results clearly indicate that the triacylglycerols consisting of Ln decrease more rapidly than the ones including saturated fatty acids and that the higher amount of these triacylglycerols in FCV result from its short curing process. While previous reports did not mention the change of triacylglycerols during the curing process, other than the change of glycolipids or the fatty acids hydrolyzed from crude lipids in tobacco leaves (32, 33), the current research first clarifies that triacyl-glycerols in tobacco leaves show the same tendency as the glycolipids and fatty acids.

CONCLUSIONS

In this research, the analysis of triacylglycerols in tobacco leaves by NARPC for separation and APCI-MS for detection enabled us to identify for the first time 35 types of triacylglycerols consisting of Ln, L, O, P and S in tobacco leaves and to introduce the proportional values for quantification to estimate actual quantitative amounts. The feasibility of the procedure using LC/APCI-MS for tobacco leaves was validated by quantification of several authentic components. The results of quantification show that the total amount and composition of triacylglycerols in tobacco leaves mainly differed between flue-cured and air-cured types. This is consistent with a previous report about the changing amounts of glycolipids and phospholipids during the curing process.

Moreover, the statistical analysis (PCA) enabled us to determine two crucial factors to classify various tobacco leaves and to confirm the gradual decrease of triacylglycerols including Ln during the curing process. The author believes that further research concerning triacylglycerols and their derivatives in various tobacco leaves and the detailed differences between the metabolisms of lipid components in tobacco leaves will be of great importance to understand physiological activities in tobacco leaves.

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