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Beiträge zur Tabakforschung International
Contributions to Tobacco Research
Volume 27 · No. 2 · April 2016
DOI:10.1515/cttr-2016-0009

A Simultaneous Analytical Method to Profile Non-Volatile Components with Low Polarity Elucidating Differences Between Tobacco Leaves Using Atmospheric Pressure Chemical Ionization Mass Spectrometry Detection*

by

Naoyuki Ishida

Product Technology Development Center, Japan Tobacco Inc., 1-17-7 Yokokawa, Sumida-ku, Tokyo, Japan

SUMMARY

A comprehensive analytical method using liquid chromatography atmospheric pressure chemical ionization mass spectrometry detector (LC/APCI-MSD) was developed to determine key non-volatile components with low polarity elucidating holistic difference among tobacco leaves. Non-aqueous reversed-phase chromatography (NARPC) using organic solvent ensured simultaneous separation of various components with low polarity in tobacco resin. Application of full-scan mode to APCI-MSD hyphenated with NARPC enabled simultaneous detection of numerous intense product ions given by APCI interface. Parameters for data processing to filter, feature and align peaks were adjusted in order to strike a balance between comprehensiveness and reproducibility in analysis. 63 types of components such as solanesols, chlorophylls, phytosterols, triacylglycerols, solanachromene and others were determined on total ion chromatograms according to authentic components, wavelength spectrum and mass spectrum. The whole area of identified entities among the ones detected on total ion chromatogram reached to over 60% and major entities among those identified showed favorable linearity of determination coefficient of over 0.99. The developed method and data processing procedure were therefore considered feasible for subsequent multivariate analysis. Data matrix consisting of a number of entities was then subjected to principal component analysis (PCA) and hierarchical clustering analysis. Cultivars of tobacco leaves were distributed far from each cultivar on PCA score plot and each cluster seemed to be characterized by identified non-volatile components with low polarity. While flue-cured Virginia (FCV) was loaded by solanachromene, phytosterol esters and triacylglycerols, free phytosterols and chlorophylls loaded Burley (BLY) and Oriental (ORI) respectively. Consequently the whole methodology consisting of comprehensive method and data processing procedure proved useful to determine key-components among cultivars of tobacco leaves, and was expected to additionally expand coverage that metabolomics study has ensured. [Beitr. Tabakforsch. Int. 27 (2016) 60–73]

ZUSAMMENFASSUNG

Zur Bestimmung der wesentlichen nicht-flüchtigen Bestandteile mit geringer Polarität wurde eine umfassende Analysemethode mit Massenspektrometrie-Detektor mit chemischer Ionisation bei Atmosphärendruck und Flüssigchromatographie (LC/APCI-MSD) entwickelt, um globale Unterschiede zwischen Tabakblättern zu erklären. Die nicht-wässrige Umkehrphasen-Chromatographie (NARPC) mit organischem Lösungsmittel sorgte für eine gleichzeitige Trennung verschiedener Bestandteile mit geringer Polarität im Tabakharz. Die Anwendung eines Vollscans auf APCI-MSD kombiniert mit NARPC ermöglichte die gleichzeitige Bestimmung zahlreicher intensiver Produkt-Ionen durch die APCI-Grenzfläche. Die Parameter für die Datenverarbeitung zur Filterung, Darstellung und zum Abgleich von Peaks wurden angepasst, um ein ausgewogenes Verhältnis zwischen Vollständigkeit und Reproduzierbarkeit in der Analyse zu erreichen. 63 Arten von Bestandteilen wie

*Received: 27th October 2015 – accepted: 29th February 2016

Solanesole, Chlorophylle, Phytosterine, Triacylglycerine, Solanachrome und andere wurden in Totalionen-chromatogrammen bestimmt, entsprechend nach authentischen Bestandteilen, Wellenlängenspektrum und Massenspektrum. Der gesamte Bereich der identifizierten Entitäten von allen Entitäten, die im Totalionenchromatogramm bestimmt wurden, erreichte über 60%, und die wesentlichen Entitäten von den identifizierten zeigten eine positive Linearität des Bestimmungskoeffizienten von über 0,99. Die entwickelte Methode und das Datenverarbeitungsverfahren wurden daher für die anschließende multivariate Analyse als durchführbar erachtet. Die Datenmatrix, bestehend aus einer Reihe von Entitäten, wurde dann einer Hauptkomponentenanalyse (PCA) und einer hierarchischen Clusteranalyse unterzogen. Die Tabakblättersorten waren auf dem PCA-Score-Plot weit von jeder Sorte verteilt, und jedes Cluster schien durch identifizierte nicht-flüchtige Bestandteile mit geringer Polarität gekennzeichnet zu sein. Während flue-cured Virginia (FCV) mit Solanachromen, Phytosterinestern und Triacylglycerinen geladen waren, waren Burley (BLY) bzw. Orienttabak (ORI) mit freien Phytosterinen und Chlorophyllen geladen. Die gesamte Methodik bestehend aus umfassender Methode und Datenverarbeitungsverfahren erwies sich als nützlich für die Bestimmung der Hauptkomponenten bei Tabakblattsorten. Sie wird außerdem voraussichtlich den Abdeckungsbereich von Metabolomikstudien weiter erhöhen. [Beitr. Tabakforsch. Int. 27 (2016) 60–73]

RESUME

Une méthode analytique complète utilisant un détecteur LC/APCI-MSD (chromatographie en phase liquide et spectrométrie de masse par ionisation chimique à pression atmosphérique) fut développée dans le but de détecter les principales composantes non-volatiles à faible polarité qui mettent en lumière les différences globales parmi les feuilles de tabac. Le recours à la chromatographie en phase inverse non aqueuse avec un solvant organique garantit une séparation simultanée de diverses composantes à faible polarité dans la résine de tabac. L'application du mode balayage complet pour l'APCI-MSD couplée à la chromatographie en phase inverse non aqueuse permit la détection simultanée de nombreux ions fragments intenses et visibles sur l'interface APCI. Les paramètres de traitement des données en vue du filtrage, de la caractérisation et de l'alignement des pics furent ajustés afin d'établir un équilibre entre l'exhaustivité et la reproductibilité de l'analyse. Soixante-trois types de composantes tels que des solanésols, des chlorophylles, des phytostérols, des triacylglycérols, des solanachromènes et autres furent identifiés sur les chromatogrammes d'ions totaux sur la base de leur authenticité, leur spectre de longueur d'ondes et leur spectre de masse. La zone complète des entités identifiées parmi toutes les entités détectées sur le chromatogramme d'ions totaux dépassa les 60% et les entités principales parmi les entités identifiées présentèrent une linéarité favorable des coefficients de détermination allant au-delà de 0,99. La méthode mise au point et la procédure de traitement des données furent, dès lors, jugées applicables à une analyse multivariée ultérieure. Une matrice de données composée d'un certain nombre d'entités fut alors soumise à une analyse en composantes principales (ACP) et à une analyse par segmentation hiérarchique. Les cultivars de feuilles de tabac se répartirent à distance les uns des autres sur la représentation graphique des scores ACP et chaque segment sembla se caractériser par des composantes non-volatiles à faible polarité identifiées. Alors que le tabac jaune de Virginie (FCV) était chargé de solanachromènes, esters de phytostérol et de triacylglycérols, le tabac Burley (BLY) et le tabac d'orient (ORI) étaient respectivement chargés de phytostérols libres et de chlorophylles. Par conséquent, l'ensemble de la méthodologie associant une méthode complète à une procédure de traitement des données s'avéra utile à la détermination des principales composantes parmi les cultivars de feuilles de tabac. Il est attendu de cette méthode qu'elle élargisse, en outre, la couverture assurée par l'étude métabolomique. [Beitr. Tabakforsch. Int. 27 (2016) 60–73]

ABRREVIATIONS

AIA: analytical instrument association; APCI: atmospheric pressure chemical ionization; CE: capillary electrophoresis; GC: gas chromatography; GC/MSD: gas chromatography mass spectrometry detection; EI: electron beam ionization; ESI: electrospray ionization; HPLC: high pressure liquid chromatography; LC: liquid chromatography; LC/ESI-MSD: liquid chromatography electrospray ionization mass spectrometry detection; MSD: mass spectrometry detection; NARPC: non-aqueous reversed-phase chromatography; PCA: principal component analysis; PDAD: photo diode array detection; RSD: relative standard deviation; SAR: systematic acquired response; SIM: selected-ion monitoring; BLY: Burley tobacco; DAC: dark air-cured tobacco; FCV: flue-cured Virginia tobacco; ORI: Oriental tobacco; SAC: sun-air cured tobacco.

Triacylglycerol names are abbreviated using initial letters of fatty acid names as follows:

P: palmitic acid; S: stearic acid; O: oleic acid; L: linoleic acid; and Ln: linolenic acid. For example, glycerol esterified by palmitic acid, oleic acid, and stearic acid is abbreviated to POS, as shown in Figure 1. The initial letters are alphabetically arranged since positional isomers like sn -2 *vs.* sn -1 or -3 positions cannot be distinguished in this research.

INTRODUCTION

Tobacco leaf is a crucial commodity for tobacco production and has a wide variety of species, cultivars and types derived from various cultivation methods and curing processes throughout the world (1, 2). Such diversity has been exclusively aimed at as an analytical target to know the components elucidating their differences resulting in various tastes and aromas of tobacco products (3, 4). For this reason, numerous components such as cembranoid, labdanoid, hydrocarbon, carotenoid, lipid, alkaloid, polyphenol, carbohydrate, organic acid, cation, anion, amino acid and polymers have been qualitatively and quantitative-

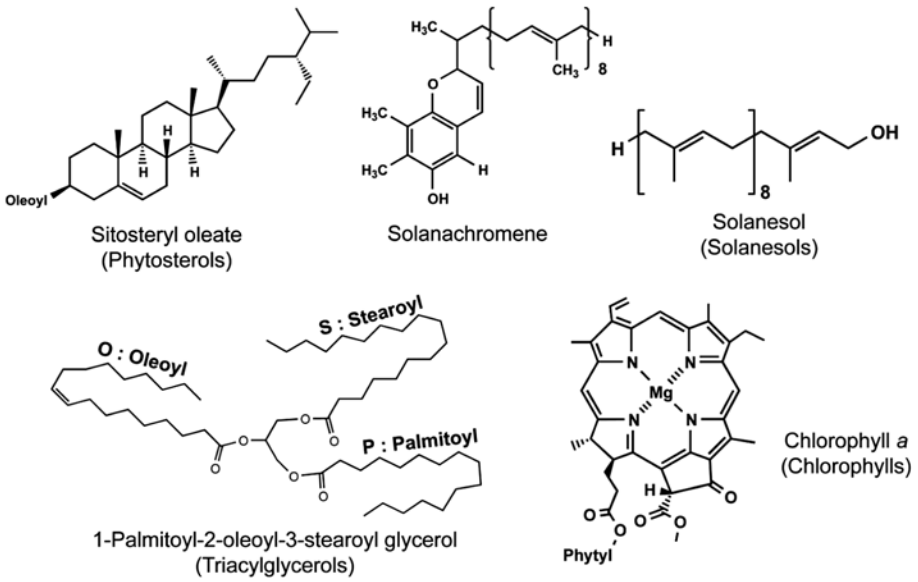

Figure 1. Non-volatile components with low polarity in tobacco leaf.

ly analyzed and reported (1, 3–6). However, those studies, in other words "target analyses", have focused on a component elucidating the difference between tobacco leaves and have remained partial knowledge within preliminary assumption of target component. Methodologies which incorporated a comprehensive analytical method simultaneously profiled numerous components, in other words "non-target analysis", to correlate differences in chemical profiles with differences in tobacco leaves have been particularly scarce in tobacco science.

Metabolomics has emerged from such demand for rapid and promising methodology that incorporates comprehensive analyses to quantitatively understand static or dynamic differences among samples of plant, animal and human metabolites. The term is nowadays described as a methodology which "seeks to identify and quantify the complete set of metabolites in a cell or tissue type and to do so as quickly as possible and without bias" (7). Metabolomics was therefore expected to become a useful methodology for tobacco leaves and has already shown the usefulness to correlate differences in components with growing districts within a nation (8, 9) and with differences resulting from systematic acquired response (SAR) caused by numerous natural stresses (10, 11). However, the methodologies applied to those studies has employed only gas chromatography mass spectrometry detection (GC/MSD) oriented to volatile components (8, 9), or liquid chromatography electrospray ionization mass spectrometry detection (LC/ESI-MSD) for components with middle to high polarity (11). Since non-volatile components with low polarity such as solanesols (12, 13), chlorophyll derivatives (14), triacylglycerols (15), solanachromene (16) and phytosterols (17) (Figure 1) are also included in tobacco leaf and have been considered unsuitable for detection in using the previously employed instruments (8–11), the metabolomics applied to tobacco leaves has yet to cover all the components in tobacco leaves.

In the first place, comprehensive analyses for metabolomics consist of a separation and detection part which are hyphenated so as to maximize comprehensiveness of method (7, 18–20). Gas chromatography (GC) for volatile components (8, 9, 21), liquid chromatography (LC) for components with low to high polarity (11, 22) and capillary electrophoresis (CE) for hydrophilic components (23) have therefore been selected in comprehensive analysis according to the chemical characters of target components. Detection for comprehensive analysis in using chromatography has basically been accomplished by mass spectrometry detection (MSD). This is because the mass spectrums make it possible to obtain the putative structures of components separated by chromatography. However, though MSD seems to be widely used, the incorporated interface has to be selected according to chemical characters of target components. Electron beam ionization (EI) in GC/MSD has been used a lot for comprehensive volatile components analysis (8, 9) due to its abundant spectrum library. Electrospray ionization (ESI) in LC/MSD has been applied to components with middle to high polarity and has become a popular interface for comprehensive analysis of peptides (24), lipids (25, 26), polyphenols (27) and all such components (28). Compared to EI and ESI, atmospheric pressure chemical ionization (APCI) has still remained a rare interface due to its specificity in low polar components (21, 29). Nevertheless the usefulness of APCI-MSD has already been presented by target analysis of non-volatile components with low polarity (13–15, 17) and the application to metabolomics was expected to provide another comprehensive method and new correlation of differences in components with differences in tobacco leaves. The objective of this study was therefore to develop a comprehensive

analytical method using LC/APCI-MSD hyphenated with non-aqueous reversed-phase chromatography (NARPC) to correlate differences in chemical profiles with differences in tobacco leaves.

In the first part of this study, a combination between APCI-MSD for detection and NARPC for efficient separation of the components with low polarity (30) is investigated to realize simultaneous detection of non-volatile components with low polarity. The developed methodology incorporating data processing is then validated in view of the number and types of detectable components in addition to reproducibility and linearity of peak areas. Data matrix consisting of featured and aligned peaks is subjected to principal component analysis (PCA) and hierarchical clustering analysis to correlate differences in components with differences in various tobacco leaves. The usefulness and comprehensiveness of the developed method will be evaluated in the end of study.

EXPERIMENTAL

Materials

The following types of cured tobacco leaves, *Nicotiana tabacum*, were stored in a warehouse of Japan Tobacco Inc. and were used for subsequent extraction: flue-cured Virginia (FCV) of Argentine, Brazil, China, Indonesia, Japan, Malawi, Spain, Tanzania, Thailand and Zimbabwe, harvested between 2005 and 2012; Burley (BLY) of USA, Brazil, India, Italy, Japan, Malawi, Philippines, Serbia, Thailand and Zimbabwe, harvested between 2004 and 2012; Oriental (ORI) of Albania, Bulgaria, China, Greece, Macedonia, Thailand and Turkey, harvested between 2005 and 2012; dark air-cured (DAC) of Brazil, India and Philippines, harvested between 2004 and 2006; sun-air cured (SAC) of India, harvested between 2004 and 2006. Solvents (acetone, acetonitrile and *n*-hexane) for extraction, dissolution and elution were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan), all of high pressure liquid chromatography (HPLC) grade. Components for identification were purchased and prepared by separation and synthesis as described in previous reports (13–15, 17).

Preparation of extracts for instrumental analysis

Approximately 100 g of tobacco leaves was pulverized using a CYCLOTEC 1093 Sample mill (FOSS TECATOR Inc., Höganäs, Sweden) with a 1-mm mesh. Pulverized samples were subsequently subjected to the extraction apparatus, an accelerated solvent extractor, ASE200 (Dionex Corporation, Sunnyvale, CA, USA). 2.5 g of pulverized sample was inserted in a pressure-resistant vessel (22 mL), which was then filled up with sea sand to remove the air gap. All samples were extracted with *n*-hexane one time: about 40 mL at 70 °C and 2000 psi. Extracts from 10 g of tobacco leaf (four vessels) were transferred to a 250 mL volumetric flask. The applied *n*-hexane extract (10 mL) pipetted by whole pipette was evaporated in vacuo, dissolved using acetone, and reapplied to the original volume (10 mL). The acetone solution was filtrated by Millex-LG with a 0.45 µm pore size (Millipore Corporation, Bedford, MA, USA) to remove the insoluble components in the solution. The filtrated acetone solution was placed in a tightly sealed glass amber vial in darkness (Agilent Tech., Santa Clara, CA, USA) and was subsequently injected into LC/APCI-MSD to replicate analyses three times on each sample.

Instrumental configuration for comprehensive analysis

The analytical system for comprehensive analysis of tobacco leaves comprised of an Agilent 1200 HPLC system equipped with a G1379B degasser, a G1312B binary pump SL, a G1367C Hip-ALS SL, a G1330B FC/ALS therm, a DAD G1315C and a 6130 quadrupole APCI-MSD (Agilent Tech., Santa Clara, CA, USA). For separation, an Excelpak SIL-C18/5C (250 mm × 4.6 mm I.D., 5 µm, Yokogawa Analytical Systems, Tokio, Japan (currently available from Agilent Tech.)) was used under the conditions described in Table 1.

Table 1. Conditions for separation.

Conditions for separation	Description
Mobile phase A	acetonitrile
Mobile phase B	acetone
Flow	1.0 mL/min
Gradient condition	A 100% at 0 min
	A 30% at 10 min
	A 20% at 30 min
	A 0% at 40 min
	A 0% (B 100%) holding until 55 min
Column temperature	25 °C

A photo diode array detection (PDAD) configured at 190–800 nm was used only for assignment of elusive components. An APCI-MSD for detection was used under the conditions described in Table 2.

Table 2. Conditions for separation.

Conditions for separation	Description
Polarity	positive
Capillary voltage	4000 V
Corona current	10 µA
Drying gas flow	5.0 mL/min
Drying gas temperature	350 °C
Fragmentor voltage	200 V
Nebulizer pressure	60 psi
Vaporizer temperature	500 °C
Treshold	0
Gain	1.0
Step size	0.10
Peak width	0.20 min
Scan range	100–1050 m/z of condensed form (centroid)

All the acquired data were processed and converted into analytical instrument association (AIA) file format for subsequent data processing with an installed software, Chemstation (Agilent Tech., Santa Clara, CA, USA).

Data processing and statistical analysis

The acquired AIA format files were processed using commercially available software, Reifycs Signpost™ (Reifycs Inc., Tokyo, Japan) to filter, feature and align peaks. In data processing, spot, peak and entity should be firmly defined to avoid misunderstanding according to the steps of data processing. Spot stands for an ion labeled with ion intensity and m/z. Peak means a normally distributed form with some area and retention time but the word was not used for data processing. This was because, although an entity seems to have a very similar meaning to peak, it stands for a filtered, featured and aligned spot without considering concept of baseline different from concept of peak. The parameters to filter spots (displayed as "Data loading filter" in the used software) and to feature filtered spots (displayed as "Spot detection") were configured as described in Table 3. Retention time was determined by one of the featured spots providing maximum ion intensity without merging isotopic spots. The parameters to align featured spots (in other words, entities) among samples (displayed as "peak alignment" in the used software) were configured as described in Table 3.

Table 3. Parameters for data processing.

Parameters for data processing	Description
Filter spots ("data loading filter" in the used software)	
Used retention time	8.0 to 54.0 min
Eliminated m/z	less than 300 m/z
Featured filtered spots ("spot detection"in the used software)	
Intensity cutoff mode	absolute
Intensity cutoff value	100
RT tolerance (±)	0.3 min
m/z tolerance to consolidate spots	−0.2 to 0.7 m/z
Aligned featured spots ("peak alignment" in the used software)	
Retention time separation tolerance	0.500 min
m/z separation tolerance	0.300

The given data matrix consisting of entities (filtered, featured and aligned spots) was then subjected to principal component analysis (PCA) on correlation coefficient matrix and hierarchical clustering analysis based on normalized data and Ward distance using a multivariate analysis software, JMP 11.0.0 (SAS Institute Inc., Tokyo, Japan).

RESULTS AND DISCUSSION

Method development for simultaneous detection

This study began with application of the previous combination between NARPC and APCI-MSD used for quantification of non-volatile components with low polarity in tobacco leaf to non-target analysis. Since tobacco leaves were extracted in the same manner and the extracts were separated by the same chromatography as reported (13, 15, 17), only the parameters applied to MSD for detection were optimized to obtain desirable ion intensity of solanesols (13), triacylglycerols (15) and phytosterols (17). Although chlorophylls in tobacco leaf were originally extracted by acetone (14), as the compositions of both extracts did not show a large difference, *n*-hexane was used for the analysis to consolidate extraction procedures. The upper two chromatograms of Figure 2 show some peaks of triacylglycerols (15) using the selected-ion monitoring (SIM) mode of APCI-MSD. The lower two chromatograms of Figure 2 are the same peaks as in the upper ones but were analyzed by full-scan mode of MSD employing lower injection volume and lower ion threshold. Although the lowered injection volume and the application of full-scan mode of MSD were expected to make it difficult to realize sufficient ion intensity, the simultaneous decrease of ion threshold (from 150 to 0) of MSD made up the decrease of ion intensity. The definite peaks of triacylglycerols and the other components which were quantified by SIM mode of MSD were consequently detected without regard to application of full-scan mode of MSD.

Data processing to filter, feature and align peaks

After the analytical method using LC/APCI-MSD was developed, the parameters to filter, feature and align spots using software (SIGNPOST™) were validated. Figure 3 is a plot figure of raw spots that were filtered with several threshold values and differently colored in gray scale dependent on peak intensities (configured at from 50 to 1000 as "intensity cutoff value" in the used software). Some of the components appearing in early retention time (less than 8 min) were not retained in the reversed phase column. Some of the other spots (less than 300 m/z) were considered as product ions derived from non-volatile components with low polarity. This was because APCI interface fragmented target non-volatile components with low polarity, as observed in many product ions originated from solanesol molecule (see in retention time of about 17 min). For these reasons, the parameters to filter spots were configured to eliminate retention time of from 0 to 8 min and ion molecules of less than 300 m/z. The case that parameters (displayed as "ion intensity cutoff" in the software) to filter spots were configured at 50, the number of filtered spots reached 1988. On the other hand, ion intensity cutoff configured at 1000 provided filtered spots of 169 (see Figure 3). The ion intensity cutoff was therefore configured at 100 providing 1024 filtered spots to balance between comprehensiveness and reliability of data. In addition, the ion intensity cutoff was configured at absolute value instead of relative value against maximum ion intensity within a chromatogram. This was because ion intensity filter configured at relative value tended to bring about detection of numerous noise peaks. The parameters to feature spots were configured at 0.3 min of retention time and −0.2 to 0.7 m/z to consolidate filtered spots with the same retention times and m/z. The values of these parameters were determined according to breadth of filtered peaks on chromatograms and varieties of isotopic peaks derived from non-volatile components with low polarity. The parameters to align featured spots among samples were configured at 0.5 min of retention time (displayed as "retention time separation tolerance" in the software) and at 0.3 m/z (displayed as "m/z separation tolerance")

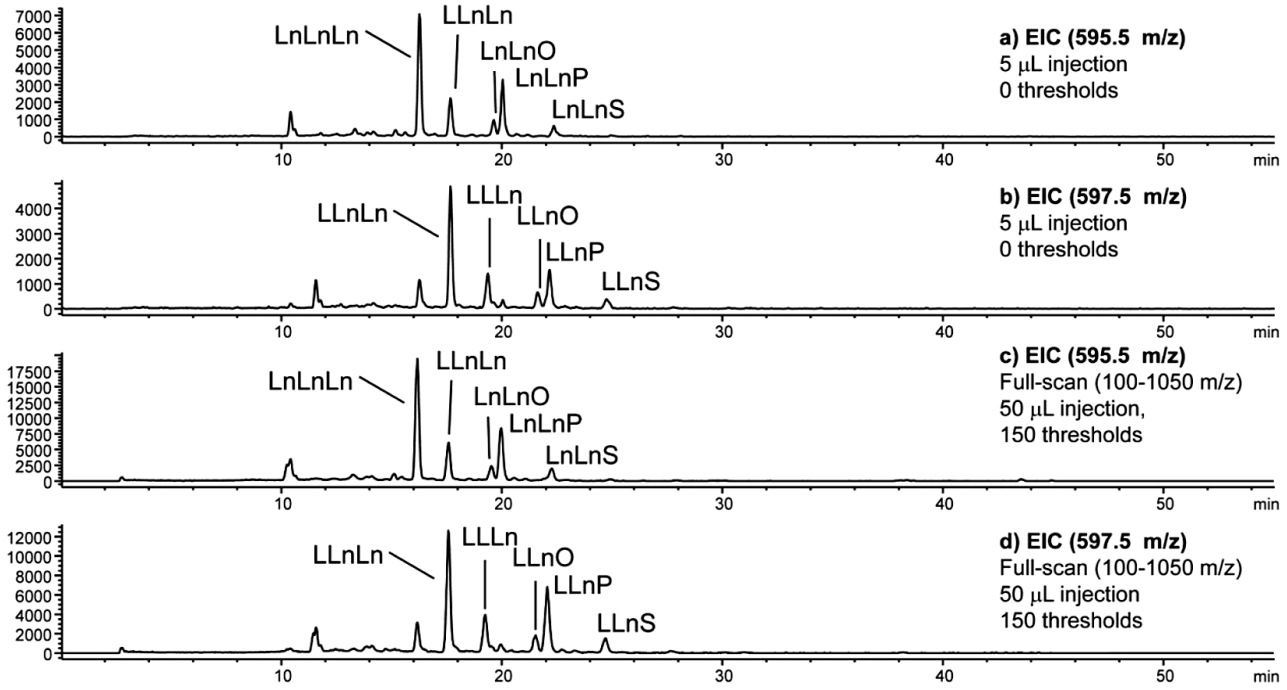

Figure 2. Comparison of chromatograms between scan and SIM mode. Figures 2a) and 2b) are chromatograms by full-scan mode with lowered injection volume and no thresholds. Figures 2c) and 2d) are those of SIM mode with larger injection volume and higher thresholds. The peaks were identified with specific diacylglycerol ions. The chromatograms are for extracts of ORI of Turkey in 2008. The analytical conditions for a) and b) are described in *Instrumental configuration*; the conditions for c) and d) are summarized in a previous report for triacylglycerols (15).

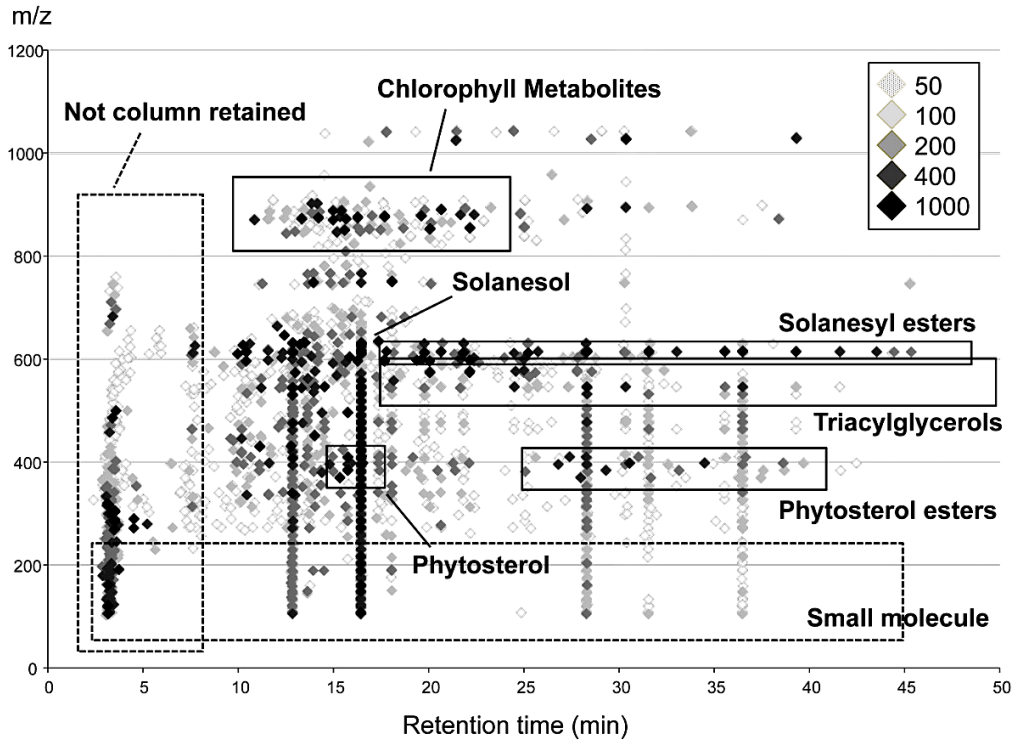

Figure 3. Plot figure of raw spots colored in grayscale. Analytical condition is described in *Instrumental configuration*. The data consisted of ten replications on one extract from ORI of Turkey in 2008 and was processed by software SIGNPOST^{TM}. Each filtered spot is colored in grayscale according to the value of intensity cutoff at a certain figure of ion abundance. The parameters to feature and align spots are described in *Data processing*.

respectively. These values to consolidate analytes were determined considering fluctuation of retention times among replicated analyses. The aligned spots were in the end used as the entities consisting of retention time and consolidated ion intensities for the subsequent validation study and multivariate analysis.

Method validation for comprehensive analysis

The developed method using LC/APCI-MSD was then validated in view of reproducibility and linearity of entities. Since deterioration of the APCI corona cone was expected to occur owing to accumulative exposure of a large amount of components derived from replicated analyses as reported (17), RSD (%) of entities obtained from consecutively or intermittently (2, 20, 60, 100) replicated analyses (three times) of a sample was weighed as shown in Figure 4.

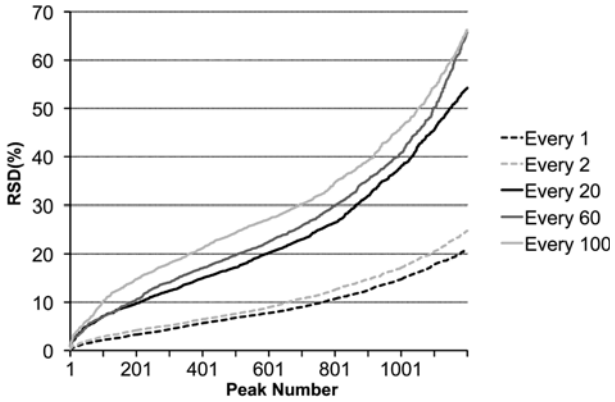

Figure 4. RSD (%) of entities according to replications. Analytical condition is described in *Instrumental configuration*. The data for RSD (%) was acquired from consecutive or intermittent analysis of ORI of Turkey in 2008. The analysis for this plot was replicated three times on one extract. The parameters to filter, feature and align spots by SIGNPOST^{TM} are described in *Data processing*.

On the whole, as intervals of replication became lowered, RSD (%) also gradually decreased (compare graphs between "every" and "every 100"). Although RSD (%) in total showed less desirable values than the reported values applying GC/MSD (9) and LC/ESI-MSD (27, 31) to metabolomics, the number of entities with favorable RSD (%) (less than 20%) reached 400 even in the case of every 100. Therefore, the obtained entities were considered sufficiently reproducible to execute subsequent multivariate analysis. Linearity of entities was calculated by analyses of incrementally diluted samples by 32 fold in the order of extract diluted by 32 to original concentrate and by subsequent replications of three times on each dilution level as summarized in Figure 5. The vertical axis of the bar chart stands for the number of entities exceeding certain determination coefficients (R^2). As the dilution rate increased, the total number of entities with favorable linearity decreased. Nevertheless, since the number of entities with favorable R^2 (over 0.99) reached more than 100 in a sample diluted by eight and any carryover of the components was not observed in the low concentrate, the developed method was considered applicable to subsequent multivariate analysis. Actual analysis for tobacco samples was replicated three times on each extract without replicating extraction of each tobacco sample. This was because previously executed quantification of solanesols (13), triacylglycerols (15) and phytosterols (17) showed smaller variance among replicated extractions than among replicated analyses on one sample.

Identification of entities

Names of entities with consolidated ion intensities are summarized in Table 4 and Figure 6. 63 non-volatile components {curly brackets indicate substances from Table 4} with low polarity were identified by wavelength spectrum, mass spectrum and standard components in addition to previous reports (13–15, 17). Solanesol {14} and solanesyl esters {40, 47, 51, 54, 55, 57, 60, 62, 63} were determined by their specific m/z (613.6 [$M+H^+-H_2O$ or acid]) and comparison of entities with authentic components (13). While areas of entities of solanesol did not show large difference among tobacco leaves in Table 4, solanesyl esters in FCV gave larger amounts than BLY and ORI. Although chlorophyll metabolites became smaller entities than the other types of components, major ones like pheophytin *a* {11}, hydroxyl pheophytin *a* {22} and solanesyl pheophorbide *a* {26} were confirmed in ORI using their spectrums and authentic components (14). Triacylglycerols were determined by specific diacylglycerol ions (15), retention times subject to the number of carbon, the unsaturated degree and authentic standard components such as LnLnLn, LLL or OOO. They were observed more in FCV and ORI than BLY. The triacylglycerols observed in chromatograms provided by developed method took

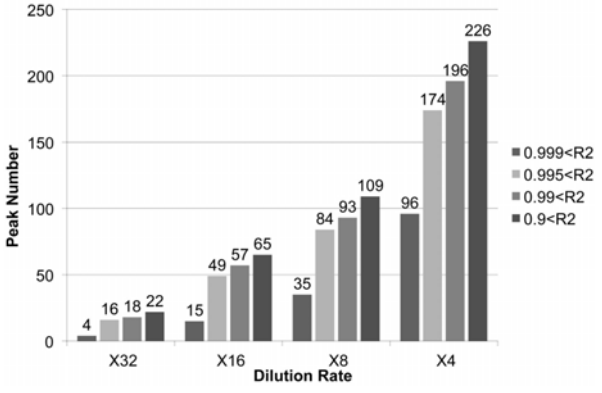

Figure 5. Number of entities according to determination coefficient and dilution rate. Analytical condition is described in *Instrumental configuration*. The data for the bar chart was acquired from analysis of extract or diluted extracts of ORI of Turkey in 2008. The analysis for this figure was executed in the order of from extract diluted by 32 to original extract and then they were replicated three times on each dilution level. The parameters to filter, feature and align spots by SIGNPOST^{TM} are described in *Data processing*.

Table 4. Determined entity with consolidated ion intensities in tobacco leaves. Analytical condition is described in *Instrumental configuration*. The analysis for this table was replicated three times on one extract. The parameters to filter, feature and align spots by SIGNPOST™ are described in *Data processing*. Assignment source stands for the tools for verification that determine elusive components.

No.	Aligned Retention Time (min)	Assigned component	Detected fragment (Ratio on base peak)	Assignment Source [a]	FCV (BRA) [b] Ave. area ± SD	BLY (BRA) [b] Ave. area ± SD	ORI (TUR) [b] Ave. area ± SD
1	12.84	Hydroxy solanesol 1 ? [c]	611.6 (100), 629.7 (36.9)	PDA, MS	358333 ± 87694	473700 ± 85483	413833 ± 48800
2	13.64	Hydroxy solanesol 2 ?	629.7	PDA, MS	n.d. [d]	n.d.	n.d.
3	13.74	Hydroxy pheophytin *b*	623.4	PDA, MS	1137 ± 424	659 ± 647	9316 ± 3935
4	13.95	Hydroxy solana-chromene ?	747.7 (100), 765.7 (10.3)	PDA, MS	131630 ± 54969	16377 ± 2932	18537 ± 2487
5	14.16	Pheophytin *b*	607.4	standard	1823 ± 403	1537 ± 1238	5542 ± 4852
6	15.08	Hydroxy pheophytin *a*	869.5	standard**	n.d.	n.d.	9898 ± 2700
7	15.29	Cholesterol	369.4	standard	8420 ± 1919	17240 ± 8610	11914 ± 6977
8	15.29	Hydroxy pheophytin *a'*	887.5 (100), 609.4 (67.9), 591.2 (69.5)	standard**	1510 ± 1020		
9	15.70	Stigmasterol	395.4, 409.4, 410.4	standard	6486 ± 2379	8132 ± 3470	5597 ± 2353
10	15.87	Campesterol	383.4	standard*	23533 ± 5331	38447 ± 4297	53893 ± 4139
11	15.87	Pheophytin *a*	871.5 (85.5), 593.3 (100)	standard	2939 ± 1281	467 ± 516	19083 ± 3693
12	16.24	LnLnLn	595.5 (99.7), 873.7 (100)	standard	26947 ± 7049	4700 ± 454	43173 ± 1891
13	16.25	Pheophytin *a'*	593.3	standard	n.d.	n.d.	n.d.
14	16.42	Solanesol	613.6 (100), 545.6 (15.8)	standard	469500 ± 74729	664133 ± 247168	406167 ± 53653
15	16.49	Sitosterol	397.4 (100), 411.4 (19.4)	standard*	30297 ± 7245	46190 ± 3863	31467 ± 4056
16	17.66	LLnLn	595.5 (48.8), 597.5 (97.0), 875.8 (100)	MS	39437 ± 9501	4453 ± 345	27780 ± 1535
17	18.05	Solanachromene	749.7 (100)	PDA, MS	150433 ± 26014	18587 ± 3001	22240 ± 1300
18	19.37	LLLn	597.5 (63.9), 599.5 (99.0), 877.8 (100)	MS	29497 ± 7865	2888 ± 396	18110 ± 942
19	19.76	Hydroxy solanesyl linolenate (C18:3) ?	611.6	PDA, MS	n.d.	n.d.	n.d.
20	20.01	LnLnP	573.5 (100), 595.5 (77.1), 851.8 (82.6)	MS	12777 ± 2515	3227 ± 516	28970 ± 5012
21	21.38	LLL	599.5 (100), 879.8 (79.5)	standard	17760 ± 3689	3430 ± 683	11256 ± 1865
22	21.43	Solanesyl hydroxy pheophorbide *a*	609.3	standard**	2597 ± 1779	3652 ± 208	13443 ± 3715
23	21.79	Hydroxy solanesyl linoleate (C18:2) ?	629.6	PDA, MS	12742 ± 3483	11207 ± 1641	10493 ± 147
24	21.83	Solanesyl hydroxy pheophorbide *a'*	591.2	standard**	n.d.	n.d.	5206 ± 4543
25	22.10	LLnP	573.5 (63.5), 575.4 (73.2), 597.5 (100), 853.8 (73.0)	MS	19250 ± 3466	2077 ± 456	18527 ± 2715
26	22.61	Solanesyl pheo-phorbide *a*	593.3	standard**	n.d.	n.d.	10176 ± 3212
27	23.52	Hydroxy solanesyl pentadecanoate (C15) ?	611.6	PDA, MS	n.d.	n.d.	n.d.
28	23.92	LLO	601.5 (100), 881.7 (6.4)	MS	n.d.	n.d.	7356 ± 5408
29	24.65	LLP (LnOP)	575.4 (40.9), 599.5 (100), 855.8 (34.3)	MS	17380 ± 4959	3352 ± 950	18627 ± 4549
30	24.65	LnOP (LLP)	573.5 (69.2), 577.5 (50.5), 599.5 (100), 855.8 (34.3)	MS	17380 ± 4959	3352 ± 950	18627 ± 4549
31	24.67	LLnS	597.5 (63.6), 601.5 (92.7), 603.5 (100), 881.8 (100)	MS	4682 ± 1662	n.d.	5214 ± 1320
32	25.04	LnPP (LnOP)	551.5 (21.8), 573.5 (100)	MS	5463 ± 1687	n.d.	12893 ± 3380
33	25.09	Hydroxy solanesyl palmitate (C16) ?	629.6	PDA, MS	6611 ± 4365	9648 ± 4044	10698 ± 5997
34	25.15	Hydroxy solanesyl oleate (C18:1) ?	611.6	PDA, MS	n.d.	n.d.	n.d.
35	27.29	LLS	599.5 (90.3), 603.5 (100)	MS	4120 ± 1120	560 ± 350	2997 ± 1108
36	27.52	LOO (LOP)	599.5 (38.4), 601.5 (100)	MS	8622 ± 1787	2516 ± 1031	7033 ± 798
37	27.52	LOP	575.4 (65.7), 577.5 (65.2), 601.5 (100)	MS	8622 ± 1787	2516 ± 1031	7033 ± 798
38	27.91	Cholesteryl linolenate (C18:3)	369.4	standard**	12877 ± 3576	4208 ± 1798	9188 ± 2899

Table 4. cont.

No.	Aligned Retention Time (min)	Assigned component	Detected fragment (Ratio on base peak)	Assignment Source [a]	FCV (BRA) [b] Ave. area ± SD	BLY (BRA) [b] Ave. area ± SD	ORI (TUR) [b] Ave. area ± SD
39	28.12	LPP (LOP)	551.5 (18.5), 575.4 (100)	MS	5282 ± 1621	1053 ± 984	4619 ± 774
40	28.25	Solanesyl linolenate (C18:3)	613.6 (100), 891.8 (2.3)	standard**	92807 ± 33807	23367 ± 20862	128840 ± 56447
41	28.71	Stigmasteryl linolenate (C18:3)	395.4	standard**	11280 ± 1315	3702 ± 2870	7060 ± 1027
42	29.24	Campesteryl linolenate (C18:3)	383.4	standard**	23367 ± 7378	7216 ± 2727	12386 ± 3718
43	30.25	Hydroxy solanesyl sitosteryl ether ? [c]	1025.9 (100), 611.6 (21.1), 545.5 (47.5)	PDA, MS	42820 ± 13052	48353 ± 22610	26213 ± 9419
44	30.47	Sitosteryl linolenate (C18:3)	397.4	standard**	31347 ± 9576	11281 ± 3521	20447 ± 7582
45	30.52	OOO	603.5	standard	n.d. [d]	n.d.	999 ± 663
46	31.07	LOS (OOP)	603.5 (100), 605.6 (59.7)	MS	2508 ± 604	2589 ± 996	1649 ± 1009
47	31.34	Solanesyl linoleate (C18:2)	613.6 (100), 545.6 (7.6)	standard**	84653 ± 39561	46350 ± 7043	32500 ± 5770
48	31.57	Cholesteryl linoleate (C18:2)	369.4	standard**	11934 ± 3563	3674 ± 1218	5086 ± 1839
49	31.75	OOP	577.5 (100), 603.5 (61.8)	MS	2290 ± 1038	2440 ± 1013	2669 ± 128
50	32.48	Stigmasteryl linoleate (C18:2)	395.4	standard**	7735 ± 3048	1749 ± 1225	1790 ± 956
51	32.96	Solanesyl myristeate (C15)	613.6 (100), 545.6 (10.5), 841.8 (1.3)	standard**	n.d.	n.d.	8952 ± 4665
52	33.05	Campesteryl linoleate (C18:2)	383.4	standard**	22913 ± 5988	5984 ± 2303	7321 ± 2205
53	34.40	Sitosteryl linoleate (C18:2)	397.4	standard**	26320 ± 10370	7204 ± 3351	11644 ± 3742
54	35.24	Solanesyl oleate (C18:1)	613.6 (100), 895.7 (0.9), 545.6 (7.3)	standard**	8689 ± 11160	3311 ± 3306	5699 ± 5854
55	36.00	Solanesyl palmitate (C16)	613.6 (100), 545.6 (7.1)	standard**	105987 ± 30638	50417 ± 44185	68960 ± 15316
56	36.19	Cholesteryl oleate (C18:1)	369.4	standard**	1890 ± 895	n.d.	1844 ± 820
57	36.55	Solanesyl margarate (C17)	613.6 (100)	standard**	n.d.	n.d.	75959 ± 102730
58	38.54	Sitosteryl oleate (C18:1)	397.4	standard**	4161 ± 2085	1022 ± 847	4483 ± 1821
59	38.58	Campesteryl palmitate (C16)	383.4	standard**	3048 ± 1478	863 ± 936	1985 ± 1313
60	39.26	Solanesyl stearate (C18)	613.6 (100), 897.8 (0.4), 545.6 (7.3)	standard**	n.d.	n.d.	n.d.
61	39.60	Sitosteryl palmitate (C16)	397.4	standard**	3990 ± 1680	1316 ± 1031	3206 ± 1425
62	42.14	Solanesyl arachidinate (C20)	613.6 (100), 925.8 (1.7), 545.6 (8.8)	standard**	14075 ± 11212	6182 ± 1758	5896 ± 3179
63	43.51	Solanesyl behenate (C22)	613.6 (100), 545.5 (6.8)	standard**	n.d.	n.d.	2965 ± 896
		Totalidentified peak areas			5383227	4995442	4103674
		Totalpeak areas (SD>1000)			8155115	7618597	7237255
		Total raw peak areas			8661262	8102758	7896941

[a] PDA: wavelength spectrum; MS: mass spectrum; standard: actual standard component. Standard components are classified into two types: standard*: commercially available component; standard**: synthesized component.
[b] Growing districts are abbreviated as follows: Brazil: BRA; Turkey: TUR.
[c] Description "n.d." in table means "not detected in this tobacco leaf".
[d] Since some components were determined only by limited spectral data, a description "?" was attached to behind their names for special notification.

forms rich in intact unsaturated fatty acid like linolenic acid and linoleic acid. Phytosterol and phytosterol esters were similarly determined by authentic components (17). While free phytosterols were observed frequently in BLY, phytosterol esters were observed frequently in FCV which were considered as similar tendency to solanesyl esters. More importantly in this study using full-scan analysis, solanachromene {17} was determined with its specific wavelength spectrum (205 and 330 nm) (13) and precursor ion (749.7 m/z) in addition to hydroxy solanachromene

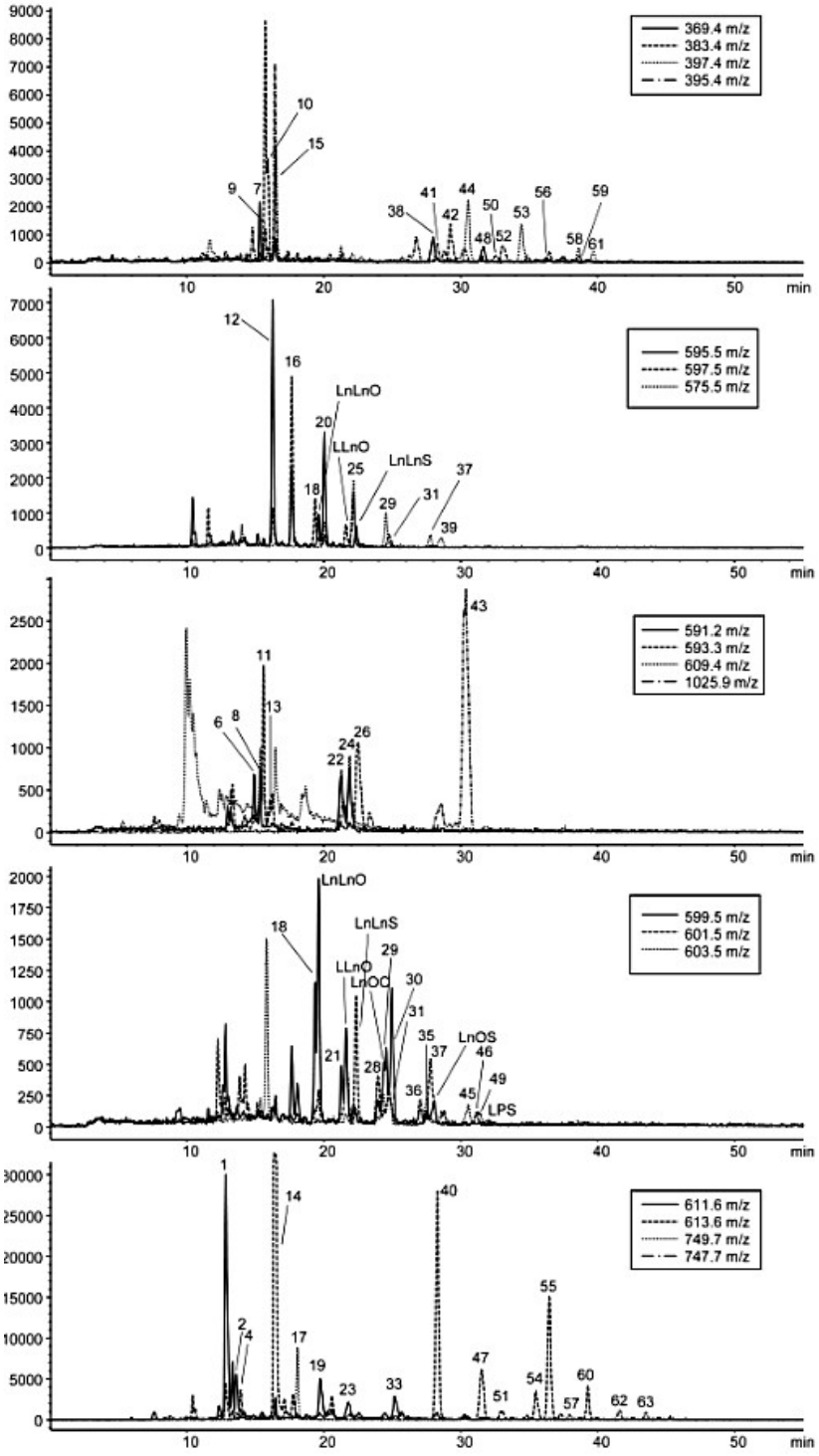

Figure 6. Identified entities of non-volatile components with low polarity on chromatogram. The chromatograms are the extracts of ORI of Turkey in 2008. All chromatograms are the extracted ion chromatograms (EIC) at a certain m/z fragmented from precursor ions in APCI interface and are overlaid to reduce the number of figures. All of the components are numbered in the same way as shown in Table 4. Analytical condition is described in *Instrumental configuration*.

(747.7 m/z) {4}. Since solanachromene was thought to generate from plastoquinone during flue-curing process (16), the fact that they were confirmed only in FCV ensured its determination. Although hydroxy solanesols {1, 2} and their esterified forms {19, 23, 27, 33, 34} were determined by its wavelength spectrum (205 nm derived from olefin moiety) and mass spectrum (629.6 m/z $[M+H^+-H_2O]$, 611.6 m/z $[M+H^+-2H_2O]$), as they seemed to take several stereoisomers due to the positional isomers of hydroxyl group and were considered less evidential than the other

entities, special notifications were added to Table 4. In the end, since the total areas of identified entities reached more than 60% (Table 4), the developed methodology was thought not to be comprehensive but favorable for profiling the components elucidating differences between tobacco leaves.

Multivariate analysis and usefulness of comprehensive analysis

Data matrix consisting of entities was subjected to principal component analysis (PCA) and hierarchical clustering analysis to correlate differences in chemical profile with differences in tobacco leaves and to evaluate its usefulness. The entities were first reduced before multivariate analysis in view of standard deviation (< 1000) of consolidated ion intensities among samples, because entities without difference among samples were considered insignificant to the purpose of this study and the total areas (consolidated ion intensities) were still retained even after its data reduction (over 90% shown in Table 4). Figure 7 shows principal component score plot on correlation coefficient matrix consisting of reduced entities. Plots are differently shaped according to cultivars of tobacco leaves. Dotted lines on the plot are clusters provided by hierarchical clustering analysis on normalized data applying ward distance. The figure showed that tobacco leaves were not clearly classified but seemed to be separated into three clusters (FCV, BLY and ORI) and sub clusters of FCV and ORI. While sub clusters of FCV1 and FCV4 were occupied with leaves harvested in Brazil and Japan, the other sub- clusters of FCV did not give any tendency regarding growing districts. BLY leaves were not dispersedly distributed differing from FCV. Non-volatile components with low polarity were thereby considered more influential on discrimination of BLY from the other cultivars. Although some clusters like ORI1 and FCV5 seemed concomitant, almost all of ORI leaves were distributed between clusters of FCV and BLY. This was probably because ORI is lightly cured tobacco leaf (sun air-cured) but its composition still retains FCV-like one (4). Samples of DAC were distributed in the middle of BLY cluster probably due to their curing processes similar to the one of BLY (air-curing process). SAC was also distributed in the position between BLY and ORI because the curing process of SAC takes similar steps to dark air-curing.

In total, the lightly cured tobacco leaves like FCV and ORI were distributed in the upper right part of the PCA plot, while the air-cured tobacco leaves like BLY, SAC and DAC were placed in the lower left.

In total, the non-volatile components with low polarity seemed to characterize cultivars and some categories of FCV. The components contributing to the observed categories were finally investigated by factor loading plot as shown in Figure 8 to know the relation of differences in chemical composition with differences in tobacco leaves. While FCV leaves were dispersedly distributed on score plot and were classified into some clusters, FCV1 was considered to be characterized by free solanesol. Similarly, FCV2 and FCV3 were characterized by phytosterol esters, triacylglycerols and solanachromene. On the other hand, chlorophylls such as pheophytin *a* and solanesyl pheophorbide *a* loaded the cluster of ORI in accordance with the previous report (14). The factor loading plot further revealed that BLY includes larger amounts of free phytosterols than the other cultivars as reported (17). Although some entities like hydroxy solanesol seemed not to load some categories, almost all the non-volatile components with low polarity were considered to characterize the cultivar of tobacco leaves. Consequently, as a proof of concept, the methodology including the simultaneous analytical method using LC/APCI-MSD was useful to profile the components elucidating differences between tobacco leaves.

CONCLUSIONS

LC/APCI-MSD was applied to a simultaneous analytical method to profile the non-volatile components with low polarity elucidating differences between tobacco leaves. The study began with the application of full-scan mode of MSD instead of SIM mode. Adjustment of injection volume and decrease of ion threshold provided favorable reproducibility of spots and ensured a sufficient number of detectable components. Raw spots were processed by software SIGNPOST^{TM} to filter, feature and align spots to give entities, and these featuring processes were then validated to execute subsequent multivariate analysis. Each entity was identified with authentic component and other spectrum data, and in the end 63 types of non-volatile components with low polarity were determined. Since total areas of identified entities reached over 60% of the total area of all entities, the data matrix consisting of all the components detected by LC/APCI-MSD were thought to be favorable to profile the components. The data matrix consisting of reduced entities was subjected to PCA and hierarchical clustering analysis in order to determine whether the entities could classify various tobacco leaves. Although the cultivar of tobacco leaves was not clearly separated on the PCA score plot, the components such as chlorophylls, solanachromene and free phytosterols seemed to characterize the observed clusters.

The developed methodology was consequently considered to be useful to provide another methodology in the metabolomics field and to relate differences in chemical profile with differences in tobacco leaves. Since some of the non-volatile components with low polarity still remained unclear and the limited number of tobacco leaves was analyzed, the use of MSD with higher resolution and the increase of samples of tobacco leaves will promote further understanding on elusive components elucidating differences between tobacco leaves, and will let this method become more robust and reliable for tobacco science.

ACKNOWLEDGEMENT

The author was supported by Japan Tobacco Inc. Technical advice from M. Yokoi and support from K. Asou is gratefully acknowledged.

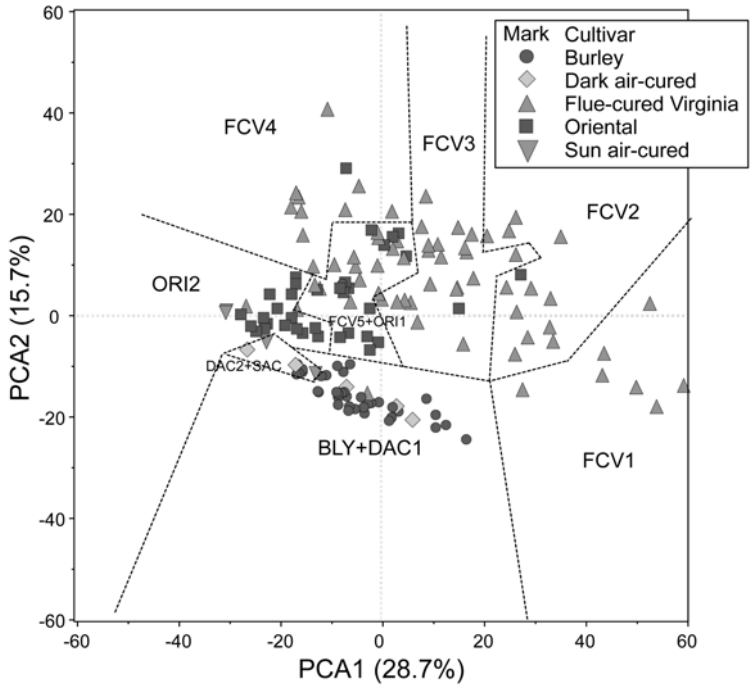

Figure 7. Score plot of PCA analysis on tobacco leaves with hierarchical clustering analysis. Analytical condition is described in *Instrumental configuration*. The analysis for the figure was replicated three times on one extract. The dotted lines are the results of hierarchical clustering analysis on data matrix consisting of reduced entities. The parameters to filter, feature and align spots by SIGNPOST^{TM} are described in *Data processing*.

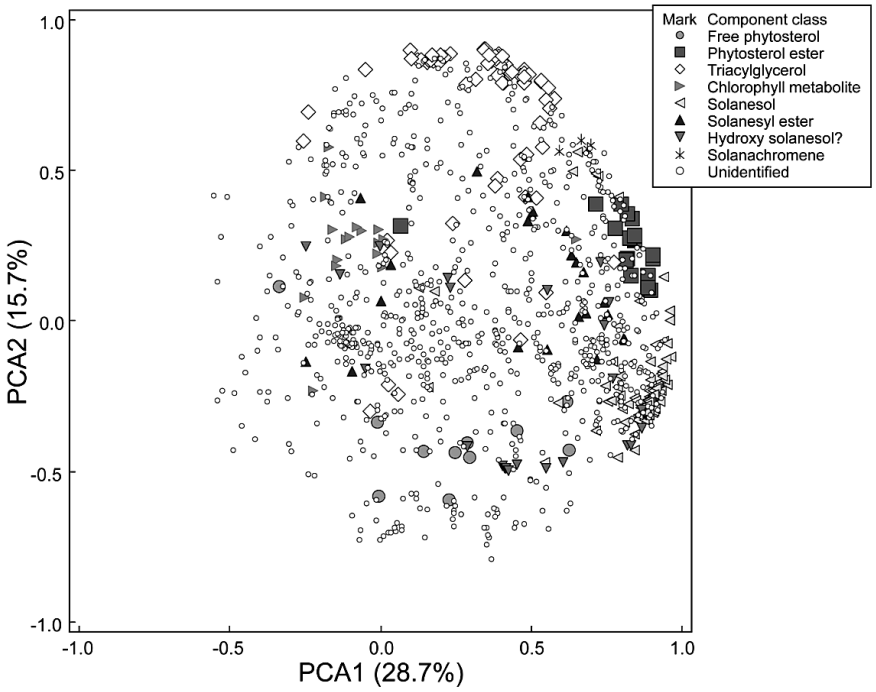

Figure 8. Factor loading plot of PCA analysis. Analytical condition is described in *Instrumental configuration*. The analysis for the figure was replicated three times on one extract. The parameters to filter, feature and align spots by SIGNPOST^{TM} are described in *Data processing*.

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Corresponding author:

Naoyuki Ishida
Japan Tobacco Inc.
1-17-7 Yokokawa, Sumida-ku, Tokyo, Japan
E-mail: naoyuki.ishida@jt.com