Talanta 115 (2013) 263–270

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Evaluation of direct analysis in real time ionization–mass spectrometry (DART–MS) in fish metabolomics aimed to assess the response to dietary supplementation

Tomas Cajka [a], Hana Danhelova [a], Antonin Vavrecka [b], Katerina Riddellova [a,*], Vladimir Kocourek [a], Frantisek Vacha [b], Jana Hajslova [a]

[a] Institute of Chemical Technology, Prague, Faculty of Food and Biochemical Technology, Department of Food Analysis and Nutrition, Technicka 3, 166 28 Prague 6, Czech Republic

[b] University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Institute of Aquaculture, Husova tr. 458/102, 370 05 Ceske Budejovice, Czech Republic

ARTICLE INFO

Article history:
Received 12 January 2013
Received in revised form
2 April 2013
Accepted 8 April 2013
Available online 15 April 2013

Keywords:
Direct analysis in real time (DART)
Mass spectrometry
Fish
Feeding
Metabolomic fingerprinting

ABSTRACT

Ambient mass spectrometry employing a direct analysis in real time (DART) ion source coupled to a medium high-resolution/accurate mass time-of-flight mass spectrometer (TOFMS) was used as a rapid tool for metabolomic fingerprinting to study the effects of supplemental feeding with cereals (triticale) on the composition of muscle metabolites of common carp (*Cyprinus carpio* L.).

First, the sample extraction and DART–TOFMS instrumental conditions were optimized to obtain the broadest possible representation of ionizable compounds occurring in the extracts obtained from common carp muscle. To this end, a simultaneous (all-in-one) extraction procedure was developed employing water and cyclohexane mixture as the extraction solvents. Under these conditions both polar as well as non-polar metabolites were isolated within a single extraction step. Next, the metabolomic fingerprints (mass spectra) of a large set of common carp muscle extracts were acquired. Finally, the experimental data were statistically evaluated using principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA).

Using this approach, differentiation of common carp muscle in response to dietary supplementation (feeding with and without cereals) was feasible. Correct classification was obtained based on the assessment of polar and as well as non-polar extracts fingerprints. The current study showed that DART–TOFMS metabolomic fingerprinting represents a rapid and powerful analytical strategy enabling differentiation of common carp muscles according to feeding history by recording metabolomic fingerprints of ionizable components under the conditions of ambient MS.

1. Introduction

Most of fish are a rich source not only of high-quality protein, essential vitamins, and minerals but also ω–3 polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [1,2]. The content of "healthy" lipids and other components in particular fish species may largely differ depending on whether the fish is wild or grown under farming conditions. In the latter case, the farming practices are based on the use of various diet supplements what typically results in higher content of neutral lipids [3]. In Central Europe, one of the most important farmed freshwater fish species is common carp (*Cyprinus carpio* L.). It is traditionally reared in earthen ponds. To increase its production, natural diet is commonly supplemented with cereals, *i.e.*, feed with high proportion of carbohydrates, representing the primary source of energy [4]. Subsequently, specific enzymatic system of fish enables to utilize high amount of carbohydrates, which are deposited in lipids. Unfortunately, their fatty acids pattern is not affected in a desirable way, nevertheless, optimization of ω–3 fatty acids composition in carps from different pond production systems can be achieved by addition of plant materials containing higher content of PUFA precursor, α-linoleic acid, ALA [5].

To monitor the effect of feeding practice on fish quality within tested experimental set-ups and to enable traceability of declared production system in case of marketed fish, suitable analytical strategies have to be available. Obtaining as much as possible comprehensive information within a short time is the current trend when choosing laboratory approach.

* Corresponding author. Tel.: +420 220 443 218; fax: +420 220 443 184.
E-mail address: katerina.riddellova@vscht.cz (K. Riddellova).

0039-9140/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2013.04.025

In general terms, traceability of the feed composition might be based on the typical metabolites pattern in animal's tissues or fluids [6]. Up to this point published studies in this research area were focused on the determination of particular metabolites classes such as carotenoids, fatty acids, vitamins, terpenes, or volatile compounds, that showed up directly or in (bio)transformed form in biotic samples taken from respective organism that was fed by specific feed. For this purpose high-performance liquid chromatography with ultra-violet detection (HPLC–UV), gas chromatography with a flame-ionization detector (GC–FID), and head-space gas chromatography–mass spectrometry (HS-GC–MS) can be employed. It should be noted that these analytical strategies were focused mainly on the analysis of a limited number of analytes mentioned above with similar physico-chemical properties [7–16].

A novel approach in this area represents metabolomics that is focused on the detection of the broadest possible range of small molecules (<1500 Da) in complex biological matrices using a single or small number of analyses. This approach has also emerged as a field of interest in food and feed analysis. Methods employing one-dimensional gas chromatography or comprehensive two-dimensional gas chromatography–mass spectrometry (1D-GC–MS, GC $\times$ GC–MS) are typically used for the analysis of (semi)volatile metabolome components and/or polar compounds (after their previous derivatization). For instance, Baumgarner and Cooper used GC $\times$ GC–MS-metabolomics platform with derivatization to detect changes in polar and non-polar metabolites during starving of rainbow trout (*Oncorhynchus mykiss*) [17]. In addition, the metabolite profiling employing 1D-GC–MS (derivatization) of Japanese medaka (*Oryzias latipes*) juveniles exposed to malathion was evaluated [18]. Further, liquid chromatography–mass spectrometry-based methods (LC–MS) are preferred for the analysis of a wide range of not GC-amenable compounds. For very polar and ionic analytes hydrophilic interaction chromatography (HILIC) can be employed or for polar and non-polar (*e.g.*, lipids) metabolites reversed phase (ultra)-high-performance liquid chromatography [RP-(U)HPLC] is utilized. Nuclear magnetic resonance (NMR) permits the detection of a broad scope of analytes within a single run but is mainly used in the analysis of polar metabolites and typically suffers from lower sensitivity as compared to GC–MS and LC–MS [19,20]. The NMR-based metabolomic approach has recently been used to assess the effect of decreasing dietary fishmeal on the health of the finfish cobia (*Rachycentron canadum*) [21], food deprivation in juvenile rainbow trout (*O. mykiss*) [22], and the monitoring of the rearing conditions of *Sparus aurata* fish specimens [23]. In other studies the exposure of zebrafish (*Danio rerio*) to different sewage effluents [24], characterization of the metabolic actions of crude *vs.* dispersed oil in smolts of Chinook salmon (*Onchorhyncus tshawytscha*) [25], and the metabolic response of juvenile Atlantic salmon (*Salmo salar*) to long-term handling stress [26] have been documented.

Besides these instrumental platforms, ambient desorption ionization techniques represent a novel solution for metabolomic fingerprinting and profiling allowing direct sample examination in the open atmosphere, with minimal or no sample preparation requirements and remarkably high sample throughput [27–30]. Using these techniques the (instrumental) analysis time can be reduced significantly (<1 min per sample) as compared to 15–60 min GC–MS or LC–MS runs.

Direct analysis in real time (DART) ionization, which was investigated in this study, represents one of the most popular atmospheric-pressure chemical ionization (APCI)-related techniques [31]. The DART ion source has been shown to be efficient for soft ionization of a wide range of both polar and non-polar compounds. However, considering the absence of chromatographic separation prior to sample ionization, the impact of matrix effects (ion suppression) can be more significant in the case of this ambient MS technique. In spite of this possible limitation, analysis at trace-level (ng/g) of various analytes (mostly contaminants) in complex matrices (food, plasma) was reported [29]. As regards data processing, strategies including in-house macro application (Excel) or Matlab software were reported in DART–MS metabolomics-based studies prior to chemometric analysis [32,33].

Until now, several papers have been published describing various DART applications including rapid analysis of various (target) substances occurring in foodstuffs and food crops [29,34–40], but only a limited number of papers have been focused on metabolomics-based studies based on non-target approach [32,33,41–43].

In this work, the proof-of-concept of differentiation of fish muscle in response to dietary supplementation (fed with and without the supplemental feeding with cereals) was investigated. For this purpose, a rapid method for metabolomic fingerprinting of common carp (*C. carpio* L.) muscle using DART–MS has been developed followed by multivariate data analysis of the acquired data sets.

2. Materials and methods

2.1. Fish samples

Common carps (*C. carpio* L.) were reared in two earthen ponds (Horák, Baštýř) in the Fishery of Třeboň, Czech Republic. Protected geographical indication of the origin of Třeboň carp is the guarantee of quality fish. Stocking density was recalculated always on 363 pcs/ha. The growing season began on May 2009, and the fish were fed with supplementary cereals (triticale). The fish on supplementary feeding (group 1, $n=20$) were compared with a control group (group 2, $n=20$) that were fed natural food only (plankton, benthos). The genetic origins of the fish were identical, and the ages of the fish were also identical (2 years). The average live weight of all fish was 0.810 and 0.675 kg for group 1 and 2, respectively. After four months the feeding experiments were completed. The average live weights of fish were 2.59 and 2.02 kg for group 1 and 2, respectively, thus there was a 32% increase of weight of fish fed with supplementary cereals as compared to the control group during the rearing evaluation period.

2.2. Chemicals

Methanol, hexane, acetonitrile, and cyclohexane were obtained from Merck (Darmstadt, Germany). Deionized water (18 MΩ) was produced by a Milli-Q system (Millipore; Bedford, MA, USA). An aqueous ammonia solution (25%, *w*/*w*) was purchased from Penta (Chrudim, Czech Republic).

2.3. Sample preparation

An amount of 2 g of fish sample (muscle without skin) was taken into a 50 mL PP centrifugation tube, followed by the addition of 10 mL of deionized water and 10 mL of cyclohexane. After a brief shaking the content was homogenized using an Ultra-turrax macerator at 5000 rpm for 1 min. The tube was finally centrifuged (10,000 rpm) for 10 min. Both cyclohexane and aqueous layers were collected using a Pasteur pipette into glass vials for subsequent DART–TOFMS analysis.

During the sample preparation, blanks were also prepared consisting of all the steps mentioned above except for the addition of sample.

2.4. DART–TOFMS analysis

For DART–TOFMS analyses, the system consisted of a DART-100 ion source (IonSense, Danvers, MA, USA), an AccuTOF LP medium high-resolution/accurate mass TOF mass spectrometer [JEOL (Europe) SAS, Croissy sur Seine, France], and an AutoDART HTC PAL autosampler (Leap Technologies, Carrboro, NC, USA). For the mass drift compensation needed for accurate mass measurement, polyethylene glycol with an average molecular weight of 600 g mol^{-1} (Sigma-Aldrich, Steinheim, Germany) at a concentration of 200 μg mL^{-1} in methanol was introduced using a Dip-it sampler (IonSense, Saugus, MA, USA) at the end of each analysis. The mass resolving power for *m/z* 128.0 (pyroglutamic acid) and *m/z* 876.8 [TAG(52:2)] was 3100 and 5800 fwhm (full width at half maximum), respectively.

MassCenter (JEOL) software (v. 1.3.0) was used for instrument control, data acquisition, and data processing. Mass spectral data were obtained by averaging of the mass spectra recorded during the exposure of the sample to the DART gas beam; background ions were subtracted and a mass drift was corrected.

2.4.1. DART(+) ionization (analysis of cyclohexane extracts)

Ion mode: positive; helium flow-rate: 4 L min^{-1}; needle voltage: 3000 V; discharge electrode: +150 V; grid electrode: +250 V; gas beam temperature: 350 °C; sampling time: 30 s; dopant: aqueous ammonia solution.

2.4.2. DART(−) ionization (analysis of aqueous extracts)

Ion mode: negative; helium flow-rate: 3 L min^{-1}; needle voltage: 3000 V; discharge electrode: −150 V; grid electrode: −350 V; gas beam temperature: 250 °C; sampling time: 5 s.

2.4.3. TOFMS detection

Mass range: *m/z* 50–1000; peaks voltage: 900 V; detector voltage: –2400 V (positive ion mode); +2200 V (negative ion mode); acquisition rate: 5 spectra s^{-1}.

2.5. Data analysis

Chemometric analysis included multivariate data analysis using (unsupervised) principal component analysis (PCA) and (supervised) orthogonal partial least squares discriminant analysis (OPLS-DA) employing the software SIMCA (v. 13.0, 2011, Umetrics, Umea, Sweden; www.umetrics.com).

In the first stage of the data processing, the raw data obtained on fish samples (40 samples × 41 selected signals in positive ion mode, 40 samples × 25 selected signals in negative ion mode) in the form of absolute peak intensities (area) were pre-processed using constant row sum, that is, each variable was divided by the sum of all variables for each sample (mass spectrum); this procedure transformed all the data to a uniform range of variability. Subsequently, log transformation and Pareto scaling were applied for particular "normalized" data sets prior PCA and OPLS-DA.

The quality of the models was evaluated by the goodness-of-hit parameter (R^2X), the proportion of the variance of the response variable that is explained by the model (R^2Y) and the predictive ability parameter (Q^2), which was calculated by a 7-round internal cross-validation of the data using a default option of the SIMCA software. In addition, the models were also evaluated in terms of recognition and prediction abilities. Recognition ability represents the percentage of samples in the training set, which were correctly classified. Prediction ability is the percentage of samples in the test set correctly classified by using the model developed during the training step. For this purpose a 4-fold internal cross-validation was used.

3. Results and discussion

3.1. Optimization of sample preparation and DART settings

Within the first phase of this study, we focused on the sample preparation and optimization of DART–TOFMS conditions for the analysis of the broadest possible spectrum of metabolites/small molecules isolated from the fish muscle. Being aware that the compounds largely differing in their physico-chemical properties may occur in fish muscle, we tested several non-discriminative approaches for their efficient isolation.

To speed up the analysis and achieve some fractionation of sample components, we employed an "all-in-one" extraction principle using both non-polar and polar solvents simultaneously. While non-polar solvent is suitable for the isolation of non-polar metabolites (*e.g.*, hydrocarbons, triacylglycerols, cholesterol, free fatty acids), the use of polar solvent, on the other hand, promotes the isolation of polar analytes (*e.g.*, amino acids, peptides, organic acids, sugars). During the course of our experiments, 2 g of fish muscle were homogenized using an Ultraturrax with 10 mL of polar solvent (water or a methanol/water mixture (1:1, v/v)) and 10 mL of non-polar solvent (hexane or cyclohexane). After separation of solvent layers by centrifugation, the obtained extracts were analyzed using DART–TOFMS, in both positive and negative ion mode at different experimental conditions (see below). No significant differences in the DART–TOFMS fingerprints were observed for polar extracts prepared using water, methanol, or a methanol/water mixture (1:1, v/v). On this account, for the isolation of polar compounds, water (as a cheap solvent) was used for subsequent experiments. Comparable extraction efficiency was also observed for the non-polar solvents. In the final method, cyclohexane was selected due to its lower volatility as compared to hexane. This factor was beneficial during DART–TOFMS analyses since after the transfer of the organic extracts (typically 12 per run) in the sampling holes of a deep well micro-plate, the spontaneous evaporation of cyclohexane was slower as compared to hexane.

The relationship between the setting of various DART operating parameters and features of mass spectra generated under particular conditions was also investigated. Helium beam temperature, flow rate, desorption time, and the use of dopant were the major parameters affecting DART ion formation and transmission into MS.

The impact of gas beam temperature was monitored at temperatures of 150, 250, and 350 °C. A temperature of 250 °C provided the highest responses for polar extracts in DART(+) and DART(−) modes and non-polar extracts in DART(−), while the much higher temperature (350 °C) was needed in the case of non-polar extracts in DART(+). In this particular case the ionization efficiency was further improved by means of using dopant (ammonia vapors). Helium flow-rate was also observed to have an influence on the DART–TOFMS responses of target analytes. This parameter was tested for 3.0, 3.5, and 4.0 L min^{-1}. The number of metabolites detected increased with increased flow rate but the rates >3 L min^{-1} led in the case of polar extracts to the dispersion of the sample on the sample stick and to splitting solvent droplets off towards the inlet orifice of the mass spectrometer, causing contamination. Therefore, for subsequent experiments, a helium flow-rate of 3.0 L min^{-1} and 4.0 L min^{-1} were used for polar and non-polar extracts, respectively.

Another important factor optimized was (thermal) desorption time. To improve not only the throughput of analyses but also the

repeatability of DART measurements, a commercial autosampler device (AutoDART-96) was used. The tested values of (thermal) desorption time included 1, 2, 5, 10, and 30 s. It was observed that 5 s provided sufficient intensity of ions (analysis of polar extracts in DART(+) and DART(−), and non-polar extracts in DART(−)), while 30 s desorption time was needed during the analysis of non-polar extracts in DART(+).

Since the analysis of the polar extracts in DART(−) and non-polar extracts in DART(+) provided the most complex fingerprints (for more details see Section 3.2), only these two measurements (mass spectral records) were used in a subsequent analysis of a large series of fish samples.

3.2. Interpretation of DART–TOFMS mass spectra

During the DART(+) analysis of polar extracts, three dominating ions were observed: *m/z* 156.1 (histidine), *m/z* 227.1 creatine, $[2M-2H_2O+H]^+$ and *m/z* 114.1 representing the sum of creatinine and creatine; the latter one undergoes degradation ($-H_2O$) to creatinine during DART desorption at high temperature (Fig. 1-A). The DART(−) spectra of the same extracts contained more signals with dominating deprotonated molecules of histidine (*m/z* 154.1 and its dimer *m/z* 309.1) followed by intensive signal of pyroglutamic acid (*m/z* 128.0), succinic acid (*m/z* 117.0), glutamine (*m/z* 145.1), glucose (*m/z* 179.0) as well as less intensive ions corresponding to di- and tripeptides (according to elemental composition) in the higher mass range (*m/z* 200–400) (Fig. 1-B). Similarly to DART(+), the ion *m/z* 112.1 represents the sum of creatinine ($[M-H]^-$) and creatine ($[M-H_2O-H]^-$).

In DART(+) with the support of dopant (ammonia), triacylglycerols were the most intensive ions (Fig. 1-C). Typical DART(+)–TOFMS spectra of TAGs are characterized by molecular adduct ions $[M+NH_4]^+$ and fragment ions of TAGs, which are, in line with expectation, the main components in fish non-polar fraction. The formation of TAG ammonium adducts was induced by ammonia vapors present in the sampling area. It should be noted that signals of $[M+NH_4]^+$ were of significantly higher intensities (approximately by 1 order of magnitude) as compared to the intensities of protonated molecules $[M+H]^+$, which were observed when the dopant solution (aqueous ammonia) was excluded from the experimental set-up. Unequivocal identification of TAGs detected in the samples and/or resolution among their stereoisomers was not possible with the DART–TOFMS technique due to the simultaneous desorption/ionization of all present compounds. It is worth noting that for sample discrimination using the chemometric processing of recorded spectral fingerprints, the identification of respective ions is not crucial. Nevertheless, as far as more information is required, then in particular cases, the unequivocal identification of individual TAGs in examined extracts can be carried out by employing HPLC separation coupled to APCI-MS [44].

On the other hand, during the DART(−) analysis of non-polar extracts mainly free fatty acids were detected; in particular, palmitoleic acid (C16:1), *m/z* 253.2; palmitic acid (C16:0), *m/z* 255.2; linolenic acid (C18:3), *m/z* 277.1; linoleic acid (C18:2), *m/z* 279.2; oleic acid (C18:1), *m/z* 281.2; stearic acid (C18:0), *m/z* 283.3; eicosapentaenoic acid (C20:5), *m/z* 301.2; arachidonic acid (C20:4), *m/z* 303.2; docosahexaenoic acid (C22:6), *m/z* 327.2; docosapentaenoic acid (C22:5), *m/z* 329.2 (Fig. 1-D).

3.3. Chemometric analysis

For chemometric analysis, several potential markers (ions) of the fish extracts acquired in positive and negative ion modes were selected after careful inspection of DART–TOFMS profiles. Depending on the ion detection threshold, hundreds of signals were automatically annotated by the software in each sample. A specially developed Excel macro was used to export the target ions (in order of tens) from the raw data. The list of markers (ions, *m/z*) used for area measurements, their tentative identification, and intra-day measurement of the extraction (expressed as relative standard deviation, RSD, %; $n=6$) is shown in Tables 1 and 2. In overall, the repeatability of the extraction was better for the non-polar extracts with an average RSD of 10%, while an average RSD of 19% was achieved for the polar extracts.

Principal component analysis (PCA) applied for data assessment represents one of the most frequently used chemometric tools. One of the main attractive features of PCA is the ability to easily project particular data from a higher to a lower dimensional space and then reconstruct them without any preliminary assumptions about their distribution [45]. PCA of complete

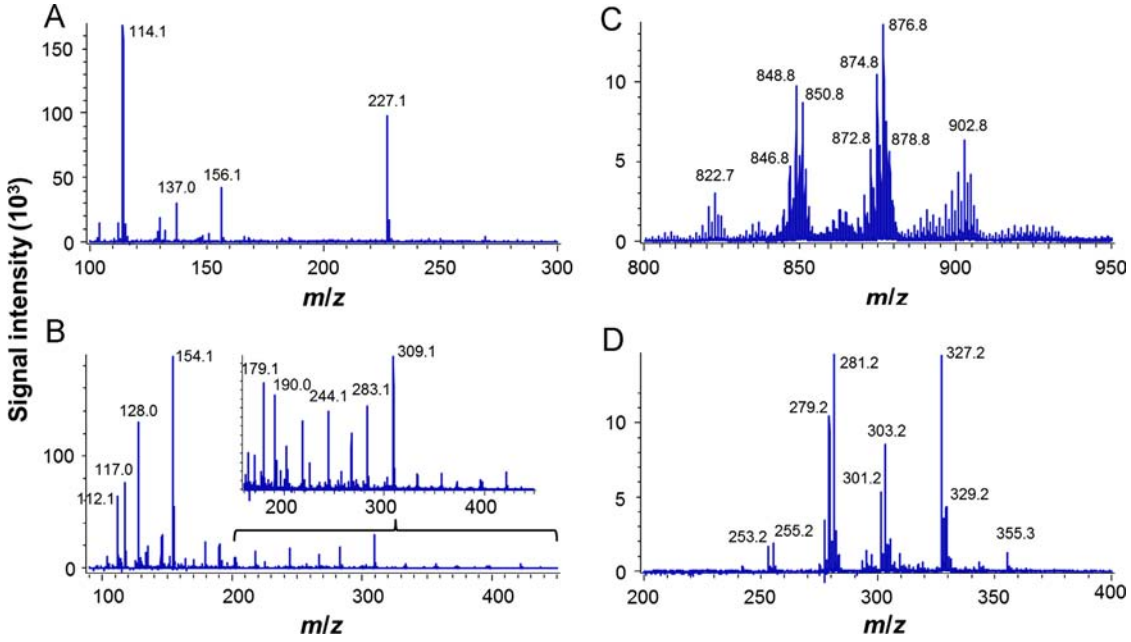

Fig. 1. DART–TOFMS fingerprints of the fish muscle extracts. (A) polar extract, DART(+); (B) polar extract, DART(−); (C) non-polar extract, DART(+); dopant: ammonia and (D) non-polar extract, DART(−).

metabolic profiles of 40 fish samples (group 1, $n=20$; group 2, $n=20$) analyzed both in DART(+) and DART(−) ion modes showed clustering behavior related to supplemental feeding with cereals (triticale). Worth to notice that in the case of histidine detected in DART(−) as deprotonated molecule (m/z 154.1) saturation of the multichannel plate detector (MCP) was commonly observed. The consequence of this phenomenon was that the top of the MS peak was cut-off resulting in the underestimation of peak area/height. This problem was solved by selecting the $[M-H+1]^-$ ion (m/z 155.1) from histidine's isotope profile. Further, due to the intensive signal of this analyte deprotonated dimer $[2M-H]^-$ (m/z 309.1) was always detected in DART(−). Since this dimer provides the same kind information as the $[M-H+1]^-$ ion, the $[2M-H]^-$ ion was excluded from the dataset prior the chemometric analysis (multicollinearity issue).

While the analysis of aqueous extracts in DART(−) provided $R^2=0.902$ and $Q^2=0.549$, some improvement of group separation was observed in the case of cyclohexane extracts measured in DART(+) with $R^2=0.920$, $Q^2=0.859$ (Fig. 2-A, -B).

Additionally, orthogonal partial least squares discriminant analysis (OPLS-DA) was constructed to identify and reveal the differential metabolites in response to supplemental feeding with cereals. In principle, the objective of OPLS is to divide the systematic variation in the X-block into two model parts, one part which models the co-variation between X and Y, and another part which expresses the X-variation that is not related (orthogonal) to Y. This may lead to better class-resolution in a discriminant problem [46]. Using OPLS-DA further improvement of separation between classes was achieved regardless what type of extracts was used. In particular, the analysis of aqueous extracts in DART(−) provided $R^2X=0.800$, $R^2Y=0.903$, $Q^2=0.807$, and again, an improvement was observed in the case of cyclohexane extracts measured in DART(+) with $R^2X=0.905$, $R^2Y=0.966$, $Q^2=0.943$ (Fig. 2-C, -D).

Using a VIP (variable importance of the projection) parameter with the threshold >1, seven markers were found to be the most significant in the polar extracts, while 14 markers were observed to be significant for non-polar extracts. The tentative identification of these markers as well as their trends (increase, ↑; decrease, ↓) for group 1 *vs.* group 2 (fish fed with *vs.* without the supplemental feeding) are summarized in Tables 1 and 2.

An additional 4-fold cross validation was performed using both types of the extracts to evaluate the performance of the OPLS-DA model. In this case, the dataset was randomly split 4-times into a calibration (training) set (3/4 of samples) with the remaining samples (1/4) being used as a test set. Using this internal validation, recognition abilities of 100% and 92.5% and prediction abilities of 100% and 87.5% were achieved for non-polar and polar extracts, respectively. To that end, differentiation of fish muscle according to diet (fed with *vs.* without the supplemental feeding) was feasible using both types of extracts analyzed by means of DART–TOFMS with better performance of the statistical model employing data from the non-polar extract analyses.

3.4. Data interpretation

In both groups of fish three main clusters of TAGs were observed: (i) TAG(50:3), TAG(50:2), TAG(50:1); (ii) TAG(52:4), TAG(52:3), TAG(52:2), TAG(52:1); and (iii) TAG(54:4), TAG(54:3), TAG(54:2). These two examined groups had the most intensive marker TAG(52:2) consisting from the main fatty acids (C16:0, C16:1, C18:1, C18:2) present in carp muscle [47]. However, the relative intensity of this TAG was higher in the group of fish fed with the supplemental feeding. Further, lower intensities of TAGs

Table 1
Analytical data of selected ions (markers) determined by DART(−)–TOFMS (analysis of aqueous fish extracts).

m/z	Ion	Elemental composition	Tentative identification	RSD, %[a]	VIP[b]	Trend[c]
104.03	$[M-H]^-$	$C_3H_6NO_3$	Serine	23		
105.02	$[M-H]^-$	$C_3H_5O_4$	Glyceric acid	16		
112.05	$[M-H]^-/[M-H_2O-H]^-$	$C_4H_6N_3O$	Creatinine/creatine	12	2	↓
114.06	$[M-H]^-$	$C_5H_8NO_2$	Proline	23	6	↑
117.02	$[M-H]^-$	$C_4H_5O_4$	Succinic acid	21	4	↑
118.05	$[M-H]^-$	$C_4H_8NO_3$	Threonine	26		
125.04	$[M-H]^-$	$C_5H_5N_2O_2$	Thymine	18		
128.03	$[M-H]^-$	$C_5H_6NO_3$	Pyroglutamic acid	11	1	↑
130.09	$[M-H]^-$	$C_6H_{12}NO_2$	Leucine	28		
132.03	$[M-H]^-$	$C_4H_6NO_4$	Aspartic acid	17		
135.03	$[M-H]^-$	$C_5H_3N_4O$	Hypoxanthine	24		
145.06	$[M-H]^-$	$C_5H_9N_2O_3$	Glutamine	21	3	↑
146.05	$[M-H]^-$	$C_5H_8NO_4$	Glutamic acid	28		
154.06	$[M-H]^-$	$C_6H_8N_3O_2$	Histidine	–		
155.06	$[M-H+1]^-$	$^{12}C_5{}^{13}CH_8N_3O_2$	Histidine	11	5	↓
161.06	$[M-H]^-$	$C_5H_9N_2O_4$	Dipeptide	7.8		
164.07	$[M-H]^-$	$C_9H_{10}NO_2$	Phenylalanine	3.7		
171.01	$[M-H]^-$	$C_3H_8O_6P$	Glycerol phosphate	20		
179.06	$[M-H]^-$	$C_6H_{11}O_6$	Glucose	17		
190.04	$[M-H]^-$	$C_7H_4N_5O_2$	Unknown	22	7	↓
202.08	$[M-H]^-$	$C_7H_{12}N_3O_4$	Dipeptide	22		
218.10	$[M-H]^-$	$C_9H_{16}NO_5$	Pantothenic acid	13		
242.18	$[M-H]^-$	$C_{13}H_{24}NO_3$	Unknown	25		
244.11	$[M-H]^-$	$C_{13}H_{14}N_3O_2$	Unknown	20		
267.10	$[M-H]^-$	$C_{12}H_{15}N_2O_5$	Dipeptide	13		
283.10	$[M-H]^-$	$C_{11}H_{15}N_4O_5$	Dipeptide	25		
309.13	$[2M-H]^-$	$C_{12}H_{17}N_6O_4$	Histidine	–		
395.19	$[M-H]^-$	$C_{18}H_{27}N_4O_6$	Tripeptide	18		

[a] Relative standard deviation (RSD) of peak area, $n=6$. For calculation, the intensities of detected ions were normalized to the intensity of the sum of all ions (constant raw sum).

[b] Variable importance of the projection; the order of markers with the VIP threshold >1.

[c] Increase (↑) and decrease (↓) of marker intensity in the group 1 (fish fed with the supplemental feeding with triticale) *vs.* group 2 (fish fed without the feed without the supplemental feeding).

Table 2
Analytical data of selected ions (markers) determined by DART(+)–TOFMS (analysis of cyclohexane fish extracts).

m/z	Ion	Elemental composition	Lipid species	RSD, %[a]	VIP[b]	Trend[c]
818.72	$[M+NH_4]^+$	$C_{51}H_{96}NO_6$	TAG(48:3)	13		
820.74	$[M+NH_4]^+$	$C_{51}H_{98}NO_6$	TAG(48:2)	5.1		
822.76	$[M+NH_4]^+$	$C_{51}H_{100}NO_6$	TAG(48:1)	7.8		
824.77	$[M+NH_4]^+$	$C_{51}H_{102}NO_6$	TAG(48:0)	14		
834.76	$[M+NH_4]^+$	$C_{52}H_{100}NO_6$	TAG(49:2)	8.8	14	↓
836.77	$[M+NH_4]^+$	$C_{52}H_{102}NO_6$	TAG(49:1)	8.4		
842.72	$[M+NH_4]^+$	$C_{53}H_{96}NO_6$	TAG(50:5)	9.1	9	↓
844.74	$[M+NH_4]^+$	$C_{53}H_{98}NO_6$	TAG(50:4)	8.9	7	↓
846.76	$[M+NH_4]^+$	$C_{53}H_{100}NO_6$	TAG(50:3)	5.9		
848.77	$[M+NH_4]^+$	$C_{53}H_{102}NO_6$	TAG(50:2)	5.8	3	↑
850.79	$[M+NH_4]^+$	$C_{53}H_{104}NO_6$	TAG(50:1)	6.0	2	↑
852.80	$[M+NH_4]^+$	$C_{53}H_{106}NO_6$	TAG(50:0)	12		
860.77	$[M+NH_4]^+$	$C_{54}H_{102}NO_6$	TAG(51:3)	14	10	↓
862.79	$[M+NH_4]^+$	$C_{54}H_{104}NO_6$	TAG(51:2)	4.3		
864.80	$[M+NH_4]^+$	$C_{54}H_{106}NO_6$	TAG(51:1)	6.9		
866.82	$[M+NH_4]^+$	$C_{54}H_{108}NO_6$	TAG(51:0)	7.9	11	↓
868.74	$[M+NH_4]^+$	$C_{55}H_{98}NO_6$	TAG(52:6)	14	8	↓
870.76	$[M+NH_4]^+$	$C_{55}H_{100}NO_6$	TAG(52:5)	12	6	↓
872.77	$[M+NH_4]^+$	$C_{55}H_{102}NO_6$	TAG(52:4)	8.9		
874.79	$[M+NH_4]^+$	$C_{55}H_{104}NO_6$	TAG(52:3)	7.1	5	↑
876.80	$[M+NH_4]^+$	$C_{55}H_{106}NO_6$	TAG(52:2)	8.2	1	↑
878.82	$[M+NH_4]^+$	$C_{55}H_{108}NO_6$	TAG(52:1)	5.6	4	↑
884.77	$[M+NH_4]^+$	$C_{56}H_{102}NO_6$	TAG(53:5)	20		
886.79	$[M+NH_4]^+$	$C_{56}H_{104}NO_6$	TAG(53:4)	19		
888.80	$[M+NH_4]^+$	$C_{56}H_{106}NO_6$	TAG(53:3)	8.5		
890.82	$[M+NH_4]^+$	$C_{56}H_{108}NO_6$	TAG(53:2)	4.5		
894.76	$[M+NH_4]^+$	$C_{57}H_{100}NO_6$	TAG(54:7)	7.8	12	↓
896.77	$[M+NH_4]^+$	$C_{57}H_{102}NO_6$	TAG(54:6)	16		
898.79	$[M+NH_4]^+$	$C_{57}H_{104}NO_6$	TAG(54:5)	13		
900.80	$[M+NH_4]^+$	$C_{57}H_{106}NO_6$	TAG(54:4)	7.5		
902.82	$[M+NH_4]^+$	$C_{57}H_{108}NO_6$	TAG(54:3)	9.1	13	↑
904.83	$[M+NH_4]^+$	$C_{57}H_{110}NO_6$	TAG(54:2)	6.3		
906.85	$[M+NH_4]^+$	$C_{57}H_{112}NO_6$	TAG(54:1)	7.8		
916.83	$[M+NH_4]^+$	$C_{58}H_{110}NO_6$	TAG(55:3)	15		
918.85	$[M+NH_4]^+$	$C_{58}H_{112}NO_6$	TAG(55:2)	5.6		
920.86	$[M+NH_4]^+$	$C_{58}H_{114}NO_6$	TAG(55:1)	4.1		
922.88	$[M+NH_4]^+$	$C_{58}H_{116}NO_6$	TAG(55:0)	14		
924.80	$[M+NH_4]^+$	$C_{59}H_{106}NO_6$	TAG(56:6)	16		
926.82	$[M+NH_4]^+$	$C_{59}H_{108}NO_6$	TAG(56:5)	10		
928.83	$[M+NH_4]^+$	$C_{59}H_{110}NO_6$	TAG(56:4)	15		
930.85	$[M+NH_4]^+$	$C_{59}H_{112}NO_6$	TAG(56:3)	10		

[a] Relative standard deviation (RSD) of peak area, $n=6$. For calculation, the intensities of detected ions were normalized to the intensity of the sum of all ions (constant raw sum).
[b] Variable importance of the projection; the order of markers with the VIP threshold > 1.
[c] Increase (↑) and decrease (↓) of marker intensity in the group 1 (fish fed with the supplemental feeding with triticale) *vs.* group 2 (fish fed without the feed without the supplemental feeding).

with PUFA [TAG(50:5), TAG(52:6), TAG(54:7)] were observed in the group of fish fed with supplementary cereals (triticale). Both these observations were in line with the results published by Vacha et al. [16], who reported higher % of fat consisting from saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) in common carp fed with supplementary cereals (triticale, wheat, corn) and lover levels of PUFAs. Worth to notice that in this study Soxhlet extraction followed by derivatization of fatty acids released from particular TAGs with subsequent GC separation with flame-ionization detection (FID) was employed.

The presence of even minor TAGs with odd-chain fatty acids is not surprise since the carp lipids may contain up to 5.3% of these fatty acids (mainly C15:0, C15:1, C17:0, C17:1) [47]. Lower intensities of these TAGs [TAG(49:2), TAG(51:0), TAG(51:3)] were observed in the group fed with supplemental feeding. It should be noted that contrary to commonly used method based on analysis of fatty acids released from TAGs by hydrolysis, mass spectra obtained by DART ionization enable to learn more about the nature of fish lipids.

As regards polar fractions, the increase of relative intensities of pyroglutamic acid (linked to glutamic acid), glutamine, and proline can be explained as a result of the dietary intake from the supplementary feeding with triticale, where proteins contain these amino acids at high content [48]. On the other hand, the decrease of relative intensity of creatine can be explained by lower intake of its precursors (arginine, glycine, and methionine) from triticale's protein. The increase of relative intensities of succinic acid originating either from aspartic acid (reductive deamination) or from glucose (bacteria activity) and decrease of relative intensity of histidine (precursor of histamine formation) refer probably more to meat/fish freshness [49] rather than to the changes of the diet composition.

4. Conclusions

The novel DART–TOFMS technique enabled differentiation of fish muscle based on recording metabolomic fingerprints of ionizable compounds generated under the conditions of ambient MS. Thanks to simultaneous extraction using polar (water) and non-polar (cyclohexane) solvents, a broad range of metabolites was isolated using this approach (TAGs, amino acids, organic acids, peptides, sugars, *etc.*). In addition, high throughput of samples was achievable by means of automated introduction of sample spread on a Dip-it sampler in front of a DART ion source (< 1 min was

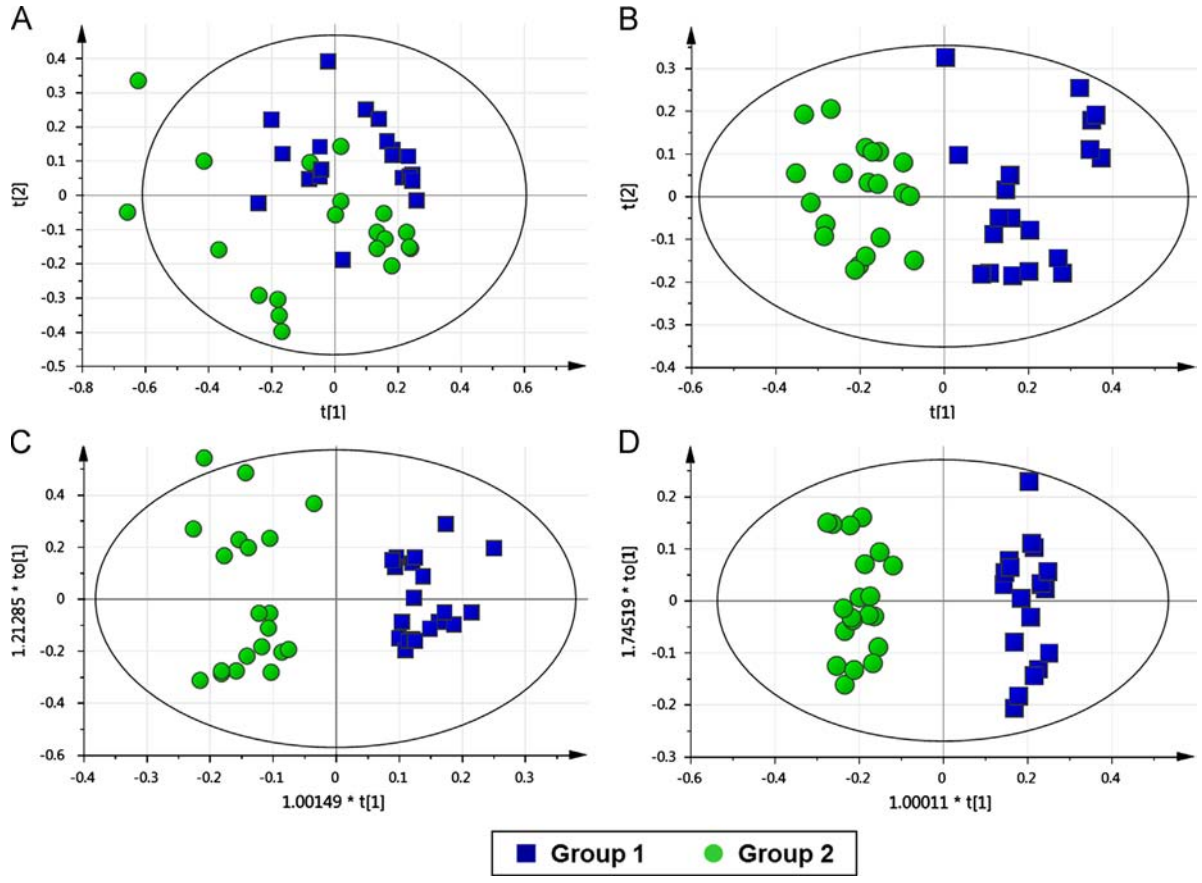

Fig. 2. A two-score plot of PCA (A, B) and OPLS-DA (C, D): (A, C) polar extracts (fish muscle); (B, D) non-polar extracts (fish muscle). (Legend: group 1=fish fed with the supplemental feeding with triticale and group 2=fish fed without the feed without the supplemental feeding).

required for a single analysis of respective sample fraction). Thus, this method benefits from its simplicity and the potential extensibility for other metabolomics-based aquaculture studies.

Using multivariate data analysis (OPLS-DA) differentiation of fish muscle according to diet [feed with and without supplemental feeding with cereals (triticale)] was feasible employing polar as well as non-polar extracts fingerprints. Relative intensity of TAG (52:2) consisting from the main fatty acids (C16:0, C16:1, C18:1, C18:2) was higher in the group of fish fed with the supplemental feeding while lower intensities of TAGs with PUFA [TAG(50:5), TAG (52:6), TAG(54:7)] were observed in the same group. Also, an increase of relative intensities of pyroglutamic acid, glutamine, and proline was observed in the group of fish fed with the supplemental feeding as compared to the group of fish fed with natural food only (plankton, benthos).

Disclaimer

Mention of brand or firm names in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the Institute of Chemical Technology, Prague and the University of South Bohemia, Ceske Budejovice.

Acknowledgments

The financial support of the Ministry of Education, Youth and Sports of the Czech Republic (Project COST Action 867) and the Ministry of Agriculture of the Czech Republic (Project NAZV-QI91B306) is acknowledged.

References

[1] ESFA Scientific Opinion, EFSA J. 2078 2011 1–30 ⟨http://www.efsa.europa.eu/en/efsajournal/doc/2078.pdf⟩ (accessed 8.01.13).
[2] EFSA Assesses Safety of Long-Chain Omega-3 Fatty Acids ⟨http://www.efsa.europa.eu/en/press/news/120727.htm⟩ (accessed 8.01.13).
[3] M.A. Hossain, Adv. J. Food Sci. Technol. 3 (2011) 455–466.
[4] L.S. Smith, Fish Nutrition, in: J.H. Halver (Ed.), Academic Press, San Diego, 1989pp. 332–339.
[5] J. Mraz, J. Machova, P. Kozak, J. Pickova, J. Appl. Ichthyol. 28 (2012) 238–244.
[6] N.Z. Ballin, Meat Sci. 86 (2010) 577–587.
[7] S. Prache, A. Priolo, P. Grolier, J. Anim. Sci. 81 (2003) 360–367.
[8] P.G. Dunne, F.P. O'Mara, F.J. Monahan, A.P. Moloney, Meat Sci. 74 (2006) 231–241.
[9] A. Priolo, A. Prache, D. Micol, J. Agabriel, J. Anim. Sci. 80 (2002) 886–891.
[10] P. French, C. Stanton, F. Lawless, E.G. O'Riordan, F.J. Monahan, P.J. Caffrey, et al., J. Anim. Sci. 78 (2000) 2849–2855.
[11] N.J. Okeudo, B.W. Moss, M.B. Chestnutt, Pak. J. Nutr. 3 (2004) 107–111.
[12] T. Moreno, A. Varela, B. Oliete, J.A. Carballo, L. Sánchez, L. Montserrat, Meat Sci. 73 (2006) 209–217.
[13] V. Vasta, J. Ratel, E. Engel, J. Agric. Food Chem. 55 (2007) 4630–4639.
[14] A.M. Descalzo, E.M. Insani, A. Biolatto, A.M. Sancho, P.T. García, N.A. Pensel, et al., Meat Sci. 70 (2005) 35–44.
[15] D.K. Larick, H.B. Hedrick, M.E. Bailey, J.E. Williams, D.L. Hancock, G.B. Garner, et al., J. Food Sci. 52 (1987) 245–251.
[16] F. Vacha, P. Vejsada, J. Huda, P. Hartvich, Aquacult. Int. 15 (2007) 321–329.
[17] B. Baumgarner, B.R. Cooper, J. Exp. Biol. 215 (2012) 1627–1632.
[18] S. Uno, A. Shintoyo, E. Kokushi, M. Yamamoto, K. Nakayama, J. Koyama, Environ. Sci. Pollut. Res. 19 (2012) 2595–2605.
[19] D.S. Wishart, Trends Food Sci. Technol. 17 (2008) 482–493.
[20] J.M. Cevallos-Cevallos, J.I. Reyes-De-Corcuera, E. Etxeberria, M.D. Danyluk, G. E. Rodrick, Trends Food Sci. Technol. 20 (2009) 557–566.
[21] T.B. Schock, S. Newton, K. Brenkert, J. Leffler, D.W. Bearden, Food Chem. 133 (2012) 90–101.
[22] A. Kullgren, L.M. Samuelsson, D.G.J. Larsson, B.T. Bjornsson, E.J. Bergman, Am. J. Physiol. Regul. Integr. Comp. Physiol 299 (2010) R1440–R1448.
[23] G. Picone, S.B. Engelsen, F. Savorani, S. Testi, A. Badiani, F. Capozzi, Nutrients 3 (2011) 212–227.
[24] L.M. Samuelsson, B. Bjorlenius, L. Forlin, D.G.J. Larsson, Environ. Sci. Technol. 45 (2011) 1703–1710.

[25] C.Y. Lin, B.S. Anderson, B.M. Phillips, A.C. Peng, S. Clark, et al., Aquat. Toxicol. 95 (2009) 230–238.
[26] T.K. Karakach, E.C. Huenupi, E.C. Soo, J.A. Walter, L.O.B. Afonso, Metabolomics 5 (2009) 123–137.
[27] A. Venter, M. Nefliu, R.G. Cooks, TrAC-Trends Anal. Chem. 27 (2008) 284–290.
[28] D.J. Weston, Analyst 135 (2010) 661–668.
[29] J. Hajslova, T. Cajka, L. Vaclavik, TrAC-Trends Anal. Chem. 30 (2011) 204–218.
[30] M.W.F. Nielen, H. Hooijerink, P. Zomer, J.G.J. Mol, TrAC-Trends Anal. Chem. 30 (2011) 165–180.
[31] R.B Cody, J.A. Laramee, H.D. Durst, Anal. Chem. 77 (2005) 2297–2302.
[32] T. Cajka, K. Riddellova, M. Tomaniova, J. Hajslova, Metabolomics 7 (2011) 500–508.
[33] M. Zhou, W. Guan, L.D. Walker, R. Mezencev, B.B. Benigno, A. Gray, F. M. Fernandez, J.F. McDonald, Cancer Epidemiol. Biomarkers Prev. 19 (2010) 2262–2271.
[34] R.L. Self, W.-H. Wu, Food Control 25 (2012) 13–16.
[35] Y. Bai, J. Zhang, Y. Bai, H. Liu, Anal. Bioanal. Chem. 403 (2012) 2307–2314.
[36] L. Vaclavik, T. Cajka, V. Hrbek, J. Hajslova, Anal. Chim. Acta 645 (2009) 56–63.
[37] L. Vaclavik, M. Zachariasova, V. Hrbek, J. Hajslova, Talanta 82 (2010) 1950–1957.
[38] L. Vaclavik, J. Rosmus, B. Popping, J. Hajslova, J. Chromatogr. A 1217 (2010) 4204–4211.
[39] J. Lojza, T. Cajka, V. Schulzova, K. Riddellova, J. Hajslova, J. Sep. Sci. 35 (2012) 476–481.
[40] H. Danhelova, J. Hradecky, S. Prinosilova, T. Cajka, K. Riddellova, L. Vaclavik, J. Hajslova, Anal. Bioanal. Chem. 403 (2012) 2883–2889.
[41] M. Zhou, J.F. McDonald, F.M. Fernández, J Am. Soc. Mass Spectrom. 21 (2010) 68–75.
[42] H. Gu, Z. Pan, B. Xi, V. Asiago, B. Musselman, D. Raftery, Anal. Chim. Acta 686 (2011) 57–63.
[43] T. Cajka, H. Danhelova, M. Zachariasova, K. Riddellova, J. Hajslova, Metabolomics, in press, http://dx.doi.org/10.1007/s11306-013-0495-z.
[44] P. Dugo, T. Kumm, A. Fazio, G. Dugo, L. Mondello, J. Sep. Sci. 29 (2006) 567–575.
[45] L.A Berrueta, R.M. Alonso-Salces, K. Heberger, J. Chromatogr. A 1158 (2007) 196–214.
[46] M. Bylesjo, M. Rantalainen, O. Cloarec, J.K. Nicholson, E. Holmes, J. Trygg, J. Chemometr. 20 (2006) 341–351.
[47] L. Kalyoncu, Y. Yaman, A. Aktumsek, Afr. J. Biotechnol. 9 (2010) 3896–3900.
[48] J. Mosse, J.C. Huet, J. Baudet, J. Cereal Sci. 7 (1988) 49–60.
[49] C.O. Chicester, E.M. Mrak, G.F. Stewart (Eds.), Advances in Food Research, vol. 16, Academic Press, New York, 1968.