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Comparative Study of Cereal Varieties by Analytical Separation Methods and Chemometrics

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any academic degree.

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Teraviljasortide võrdlev uurimus analüütiliste lahutusmeetodite ja kemomeetria abil

TUULI LEVANDI

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications:

I. T. Levandi, C. Leon, M. Kaljurand, V. Garcia-Canas, A. Cifuentes. Capillary electrophoresis time-of-flight mass spectrometry for comparative metabolomics of transgenic versus conventional maize. Anal. Chem. 2008, 80, 6329-6335.

II. T. Levandi, T. Püssa, M. Vaher, P. Toomik, M. Kaljurand. Oxidation products of free polyunsaturated fatty acids in wheat varieties. Eur. J. Lipid Sci. Technol. 2009, 111, 715-722.

III. M. Vaher, K. Matso, T. Levandi, K. Helmja, M. Kaljurand. Phenolic compounds and the antioxidant activity of the bran, flour and whole grain of different wheat varieties. Procedia Chemistry. 2010, 1, 76-82.

IV. T. Levandi, T. Püssa, M. Vaher, A. Ingver, R. Koppel, M. Kaljurand. Principal component analysis of HPLC-MS/MS patterns of wheat (*Triticum aestivum*) varieties extracts. (*accepted to the Proceedings of the Estonian Academy of Sciences*)

THE AUTHOR´S CONTRIBUTION TO THE PUBLICATIONS

The contribution by the author to the papers included in the thesis is as follows:

I, II and **IV**: carrying out all of the experiments; main person responsible for planning and writing the manuscript.

III: performed a large part of the experiments; played a minor part in writing the manuscript.

OTHER PUBLICATIONS IN RELATED FIELD

V. K. Helmja, M. Vaher, T. Püssa, A. Orav, A. Viitak, T. Levandi, M. Kaljurand. Variation in the composition of the essential oils, phenolic compounds and mineral elements of *Hypericum perforatum* L. Growing in Estonia. Nat. Prod. Res. 2011, 5, 496-510.

VI. K. Truus, M. Vaher, M. Borissova, M. Robal, T. Levandi, R. Tuvikene, P. Toomik, M. Kaljurand. Characterization of Yew Tree (*Taxus*) Varieties by Fingerprinting and Principal Component Analysis. Nat. Prod. Comm. 2012, 9, 1143-1146.

ABBREVIATIONS

amu	atom mass unit
ANOVA	analysis of variance
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
BGE	background electrolyte
Bt	Bacillus thuringiensis
CA	cluster analysis
CE	capillary electrophoresis
CEC	capillary electrochromatography
CN	correlation network
CNL	constant neutral loss
CF-FAB	continous-flow fast atom bombardment
CSF	cerebrospinal fluid
CZE	capillary zone electrophoresis
DA	discriminant analysis
DAD	diod array detector
9-oxo- DHODE	9-oxo-12,13-dihydroxy-10-octadecenoic
13-oxo-DHODE	13-oxo-9,10-dihydroxy-11-octadecenoic
9,10-DiHOME	9,10-dihydroxy-12-octadecenoic acid
12,13-DiHOME	12,13-dihydroxy-9-octadecenoic acid
DIOS	desorption ionization on silicon
DPPH	2,2-diphenyl-1-picrylhydrazyl
15,16-EODE	15,16-epoxy-12,15-octadidecenoic acid
9,10-EODA	9,10-epoxyoctadecanoic acid
EOF	electroosmotic flow
ESI	electrospray
GABA	gamma-amino butyric acid
GAE	gallic acid equivalent
GALDI	graphite-assisted laser desorption ionization
GC	gas chromatography
GMO	genetically modified organism
GSH	glutathione
^{1}H	proton (NMR)
HCA	hierarchical cluster analysis
HILIC	hydrophilic interaction liquid chromatography
9-HODE	9-hydroxy-10,12-octadecadienoic acid
13-HODE	13-hydroxy-9,11-octadecadienoic acid
9-oxo-13-HODE	9-hydroperoxy- 10,12,15-octadecatrienoic acid
13-oxo-9-HODE	13-oxo-9- hydroxy-10-octadecenoic acid

HPLC	high performance liquid chromatography
IT	ion trap
LC	liquid chromatography
LDA	linear discriminant analysis
MALDI	matrix assisted laser desorption ionization
MEKC	micellar electrokinetic chromatography
MS	mass spectrometry
NIMS	nanostructure-initiator mass spectrometry
NMR	nuclear magnetic resonance
PC	principal component
SDS	sodium dodecyl sulfate
SIM	selected ion monitoring
9,10,18-THODE	9,10,18-trihydroxy-octadecanoic acid
9,12,13-THODE	9,12,13-trihydroxy-10-octadecenoic acid

INTRODUCTION

Metabolomics focuses on high-throughput characterization of small molecule metabolites in biological matrices. Metabolomics only became possible as a result of recent technological breakthroughs in small molecule separation and identification. These include high-resolution MS instruments for precise mass determination, NMR spectrometers, HPLC, GC and CE systems for rapid compound separation, as well as new software programs to rapidly process spectral, chromatographic or electrophoretic patterns.

Also, metabolomics is called as a data driven strategy, trying to find markers of a situation under study without a priori hypothesis. It has evolved from the simple pattern recognition strategy, due to the interest for the final identification of markers responsible for class separation, i.e., from data to knowledge.

So far, most of the work in metabolomics has focused primarily on clinical or pharmaceutical applications. However, recently, metabolomics has also emerged as a field of increasing interest to food and nutrition scientists. The fact that metabolomics allows the simultaneous characterisation of large numbers of chemicals in biological systems makes it an attractive tool to gain a far more detailed and comprehensive molecular picture of food. Metabolomic applications within the food science are diverse ranging from analysing the food components; assessing the food quality, authenticity and safety; food microbiology; food processing and so forth.

Metabolic profiling of crops is increasingly becoming popular in assessing plant phenotypes and genetic diversity, studying the stress biology in plants and so on. In plant breeding identified biomarkers can be used to guide or facilitate future developments in crop improvement in terms of biochemical composition, nutritional value, tolerance to biotic and abiotic stress.

Improved varieties and high quality seeds are also basic requirements for productive agriculture. Metabolomics can potentially enable breeders to achieve high quality along with high yield.

AIMS OF THE STUDY

In the present work, the aim was to broaden the scope of "food metabolomics" for comparison of different cereal varieties, so far having attracted less attention. In more detail we aim to:

- develop sample preparation and metabolites extraction protocols for preparation cereal varieties required for analysis them by HPLC and CE.
- investigate the applicability of CE as a less used complementary/alternative separation method to the HPLC in separation of metabolites in cereals.
- demonstrate the applicability and usefulness of PCA in data reduction and extraction of the maximum useful information from the metabolic profiles whilst direct analysis of metabolic profiles of cereals is complicated mainly due to the huge amount of data that is produced in one HPLC-MS measurement.
- characterize and compare different cereal varieties.
- identify possible biomarkers in selected case studies.

1. LITERATURE OVERVIEW

1.1. Metabolomics in food science

Metabolomics, the study of `as-many-small-metabolites-as-possible` in a biological system, has become an important tool in many areas of food science (e.g. food component analysis; food quality, authenticity and safety assessment; food consumption monitoring; food processing; food microbiology). Metabolites can range from small inorganic ions to hydrophobic lipids and complex natural products, and occur in widely diverging concentrations, spanning over nine orders of magnitude (from pmol to mmol).[1-3]

The most common purposes for food analysis are determination of chemical composition (targeted/untargeted), production control, variety or strain differentiation, origin verification or differentiation, quality or safety evaluation, metabolite characterization or correlations, genetically modified organism (GMO) identification/differentiation, bacterial contamination etc. The term "metabolomics" is used broadly to cover approaches related with investigating subsets of the metabolome. It provides a detailed measurement of the phenotypic responses of living systems to genetic and environmental changes.[1]

Metabolomic analyses have been generally classified as targeted or untargeted.[1] Target analyses focuses only on a particular metabolite, or metabolite class, with most cases requiring identification and quantification. In contrast, an untargeted metabolomic focuses on the separation and detection of as many groups of metabolites as possible in a single analysis, to obtain patterns, or fingertips, without necessarily identifying, nor quantifying, individual metabolites. Based on the specific objective of the analysis and data manipulation, most metabolomics studies can also be classified as discriminative, informative, and/or predictive.[1] Discriminative analyses have been aimed at fining differences between sample populations, without necessarily creating statistical models, or evaluating possible pathways. In contrast, informative metabolomic analyses have focused on the identification and quantification of targeted or untargeted metabolites, in order to obtain sample intrinsic information. In cases of predictive metabolomics, statistical models based on metabolite profile and abundance are created to predict a variable that is difficult to quantify by other means.[1-7]

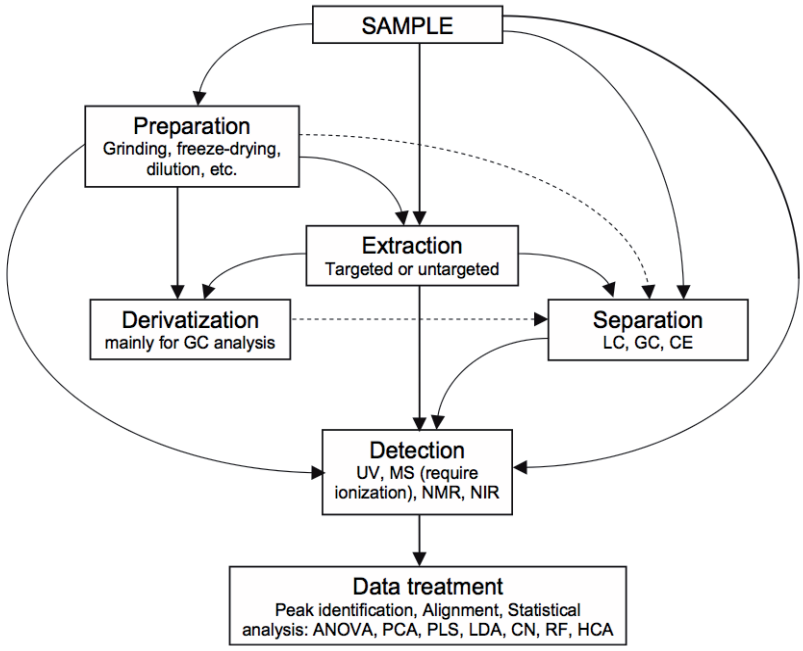

Figure 1. Schematic representation of the process of metabolomic analysis[1]. Abbreviations: see the list of abbreviations in the page 8-9.

A typical process of metabolomic analyses is presented in Figure 1.[1] The selection of the steps depends upon the type of study. Detection and data analysis are essential steps in all metabolomics studies, others are used if needed.

1.2. Metabolic profiling techniques

Metabolites include wide variations in chemical (e.g. molecular weight, polarity, solubility) and physical (e.g. volatility, charge) properties. The degree of diversity includes ionic polar compounds, such as oxalate, low molecular weight, volatile organic metabolites (urea), to the higher molecular weight, polar (carbohydrates) and non-polar (terpenoids and lipids) metabolites. In addition, concentrations also extend over an estimated 7–9 orders of magnitude in concentration (pmol–mmol).

The term "metabolic profile" was introduced in the 1970's after it was demonstrated that gas chromatography-mass spectrometry (GC-MS) could be used to measure compounds present in human urine and tissue extracts.[8] Metabolite profiling is usually defined as the identification and quantification of a selected number of pre-defined metabolites, generally related to a specific metabolic pathway(s). Sample preparation and instrumentation are employed in order to isolate those compounds of interest from possible matrix effects prior to

detection, normally with chromatographic separation prior to detection with mass spectrometry (MS).[9]

The two major analytical techniques used for metabolic profiling at this time are ^{1}H NMR spectroscopy and MS. NMR is an attractive technique for high-throughput fingerprinting and profiling studies, as it does not require extensive sample preparation, is non-destructive, and can uniformly detect all compounds with NMR-measurable nuclei. NMR is typically more robust and reproducible than hyphenated MS-based techniques, and can allow the utilization of libraries and inter-laboratory validation studies. Limitations are seen in method sensitivity, as only highly abundant metabolites will be detected and the high initial instrument acquisition cost.[4,10,11]

Constitutive levels of metabolites readily respond to perturbations in environmental conditions. NMR-based metabolite profiling, typically in conjunction with multivariate analyses, is well suited to monitor and quantify the degree of metabolic impact induced by genetics, environment, or bioengineering.[4]

In the past few years, high-resolution NMR has commonly been used for the analysis of metabolite profiles of biological fluids[12-14], plant extracts[15-18] and food component analysis[19-25].

MS has a major role in metabolite profiling, due to its sensitivity and widespread availability. MS is generally more sensitive than ^{1}H NMR, and enables the detection of metabolites that are below the NMR detection thresholds. Hyphenated MS techniques (GC-MS, liquid chromatography (LC)-MS, capillary electrophoresis (CE)-MS) have become increasingly utilized for metabolic profiling over the last few years.

There are also some issues concerning protocol and data standardization, system robustness and lack of reproducibility that can cause difficulties. As a result, MS-based metabolite profiling results are not interchangeable between researchers, and tracking back to previous analyses shows little promise for the unambiguous identification of key markers. Hence, the remaining issues mostly affect the non-targeted approaches. This means that more validation work is necessary and, in this respect, cooperation and exchange or results between different laboratories is the only way to understand the source of the problem, in order to find ways to solve it.[9,10]

MS has also been applied as a standalone analytical tool via the direct infusion of samples. This mode is ideal in population studies for the metabo-mapping of large numbers of samples; however it may face limitations in overcoming the matrix effect and in the analysis of isobaric substances[26]. Additionally, full-scan MS cannot discriminate structural isomers or enantiomers. The coupling of chromatographic methods with MS can substantially increase the metabolome depth-of-coverage, separate structural isomers, provide an additional dimension sufficient for characterization and differentiation of a large number of metabolites, including isomers. At the same time, chromatographic separation prior to ionization also decreases the number

and the chemical diversity of compounds simultaneously entering the mass spectrometer at any specific point in time. This significantly reduces matrix-ionization suppression/competitive ionization, which is considered a major limitation in direct mass analysis.[6]

Lately, there has also been considerable interest in laser desorption ionization techniques, such as MALDI and SELDI.[27,28] Although these have been developed with an aim towards large molecule analysis (e.g. proteins and polynucleotides), their application in small molecule analysis through imaging approaches may offer the advantage of analyzing selected regions of a specimen, thus providing data on the localization of the detected metabolites. Such methodologies are, at the moment, mostly used in targeted analysis, where the researcher is looking for pre-selected masses, along e.g. different areas of a tissue. To overcome the expected limitations due to the excess of signal originating from the matrix, which can obstruct detection in lower masses, matrix-free ionization techniques (GALDI, DIOS/NIMS or SIMS) have also been applied in various analytical fields, but their application in metabolomics is still very much limited.[28]

Different techniques have distinct advantages that can be exploited in investigating different metabolite classes, and the resulting information put together to obtain better characterization of the metabolome, thus, leads to the importance of complementary approaches.

A major obstacle in metabolomics continues to be the identification and quantification of unknown metabolites in complex biological samples when purified standards are unavailable. Comprehensive identification includes data acquisition, ion annotation, mass-based identification, spectral interpretation, spectral matching and metabolite verification. However, to verify the identity of an unknown metabolite confidently, the authentic standard still needs to be obtained and injected into the same instrument with the biological sample to compare their MS/MS spectra and retention times. Briefly, the most reliable way to identify a metabolite unambiguously and confidently is to compare its mass, retention time and fragmentation spectrum with those of authentic standards.[29,30]

1.2.1. Gas chromatography mass spectrometry

GC-MS is one of the most widely used analytical techniques in metabolomics[31-37]. GC–MS was applied to metabolomic studies much earlier than LC–MS. GC–MS offers high sensitivity and resolution power, excellent reproducibility, and extensive and highly reproducible fragmentation, thus providing very good identification potential due to the established databases (NIST, Wiley, Fiehn Metabolomics library). Other advantages are the ease of instrument use and low cost.

GC-MS instruments using linear quadruple analyzers have been available for decades providing a robust technology that is amenable to automation and provides high sensitivity and large dynamic range, but nominal mass accuracy and slow scan speeds. The requirement for high throughput has led to the use of

nominal GC-time-of-flight (TOF)-MS with much faster scan rates. High scan rates allow rapid temperature gradient programs, resulting in shorter run times and increased sensitivity.

A significant development in GC in the last decade is the comprehensive, two-dimensional GC (GC×GC). Generally, the sample components are separated in the first column according to their volatilities, then small fractions of the effluent are trapped and focused using a modulator, and sequentially released into the second column for further separation, this time, based on polarity differences. GC×GC offers much greater peak capacities, and with high-scan speed TOF-MS as the detection method, it is good for complex samples.

Multidimensional GC generates large and complex datasets, including the first-dimensional retention time, the second-dimensional retention time, and TOF-MS m/z values. The resultant 3D datasets are large and complex, and make manual qualitative and quantitative processing of the data from large biological experiments extremely difficult and time consuming. Several instrument-specific and independent commercial software are available for the analysis of 2D-GC data.[6,33]

GCxGC has been applied in the discovery of new metabolic biomarkers for diabet, in the separation of pathological metabolites extracts from human urine to locate and to quantify target analytes for disease profiling[31], and in the separation of metabolites from plant samples with subsequent data analysis using principal component analysis (PCA) for comparison of metabolite profiles of different species of plants[35].

However, the main drawbacks of the GC-MS technique are the sample preparation, which is tedious, time consuming and can be error prone, and the limitations caused by the requirement to deal with volatile metabolites and thermal stability. Involatile metabolites may be converted to different forms of derivatives during the derivatization reaction, thus producing a specimen where different forms of the same parent metabolite exist together. Furthermore, there is the issue of byproduct formation and degradation. It is noted that derivatization steps add time and complexity to sample preparation and introduces an additional source of variance to the experimental procedures.[6,7,10]

1.2.2. Liquid chromatography mass spectrometry

LC–MS is extensively used MS technology especially in the life science and bioanalytical sectors, due to its ability to separate and detect a wide range of metabolites. The method allows for the collection of both quantitative and structural information, and can achieve pg mL^{-1} sensitivities. LC–MS offers a very versatile tool to undertake the majority of analytical tasks in metabolite profiling studies.[10]

LC-MS analysis does not require sample derivatization, and that simplifies the sample-preparation steps as well as identification of metabolites, which can be complicated by chemical modifications of unknowns prior to GC-MS. The identification of metabolites in LC-MS is achieved through accurate-mass

determination, tandem-MS analysis, and/or coupling to NMR. The majority of metabolite profiling studies is currently performed on TOF–MS machines due to the sensitivity, rapid data acquisition and high mass accuracy (typically < 5 ppm).

A major disadvantage of LC-MS in metabolomic profiling is the lack of transferable LC-MS libraries for metabolite identifications. In MS analyses the obtained data is very complex, where three dimensions represent retention time, m/z value and signal intensity. The number of features detected in MS should not be taken as a direct measure of the number of metabolites present in the sample. Depending on the software employed for data (pre)processing, peak lists can consist of adducts, multimers, isomers or fragments leading to double or even multiple features detected per metabolite. There are some key issues to consider, e.g. LC–MS data (full scan or SIM) cannot be easily correlated or compared between different MS instruments, thus leaving few possibilities for the generation of spectral libraries, and adduct formation in LC–MS cannot be safely predicted or controlled. The safest way is to combine high mass accuracy data with database searching in available databanks, take into account the retention time and further experiments with purified fraction containing the isolated metabolite of interest by GC-MS, NMR or CE. The only conclusive and unambiguous method to identify the metabolite is the injection of authentic reference standards to verify the identification.[6,10]

The choice of ionization mode used in LC–MS analyze plays an important role in the metabolite profile that will be obtained. Certain types of molecules are better ionized in one ionization mode, or one polarity, while other molecules are ionized more efficiently in another mode. ESI in positive mode is the most common mode in LC–MS, because it can effectively ionize a wide range of medium polar and polar molecules. Negative ionization provides superior results for certain analyte classes (e.g. organic acids, polyphenols, carbohydrates). Other interfaces such as APCI or APPI are preferred for more non-polar analytes and has been used in a smaller number of metabolomic investigations.[10,38-41]

The majority of LC–MS studies for metabolite profiling employ reversed-phase (RP) gradient chromatography[40-45], normally using C18 columns. RPLC can separate semi-polar compounds (phenolic acids, flavonoids, glycosylated steroids, alkaloids and other glycosylated species). This chromatographic mode, although the most widely used and the best documented, is not really well suited for the analysis of the highly polar and/or ionic metabolites, which are often poorly retained and elute with the solvent front, thus hindering their detection with MS.

Enhanced retention and separation of polar metabolites (e.g., sugars, amino sugars, amino acids, vitamins, carboxylic acids and nucleotides) can be achieved by hydrophilic interaction liquid chromatography (HILIC) or ion-exchange[51]. In these polar columns (e.g. amino-propyl), a stagnant water layer is established within the stationary phase and the separation is achieved by partitioning the analytes between that polar layer and the mobile phase, which is aqueous-

organic based, with the consequence being that elution is in order of increasing hydrophilicity. Ion-exchange chromatography can also be used for the separation of ionic or polar compounds, but the combination of this with MS is not favorable due to the high concentrations of non-volatile salts in the mobile phase.[6,46]

The application of so-called ultra-high pressure LC (UHPLC) separations offers significant advantages that cannot be easily achieved by other technologies. UHPLC operates at relatively higher pressures (approximately 15,000 psi compared to 6000 psi for HPLC) and use columns packed with sub-2-μm stationary phases. Reduced particle sizes greatly enhance chromatographic resolution and efficiency, which provide enhanced opportunities for resolving complex biological mixtures in non-targeted metabolite profiling.[6] UPLC-MS is used increasingly in metabolomic applications.[44,45,47]

Multi-dimensional chromatography is a promising and attractive technique to increase the separation peak capacity[48-51]. In principle, fractions from the first column are regularly transferred at constant intervals to the second column via automated valves equipped with multiple sample loops. The first column is used for the primary separation mode, usually with long analysis times and gradient elution, while a second short column with larger inner diameter is used for the orthogonal separation of the modulated fractions from the first-dimensional separation. However, fast modulation is needed to minimize the loss in the first-dimensional resolution and decrease extra band broadening, which demands a very fast second-dimensional separation. Two-dimensional LC has been utilized mainly in targeted metabolite analysis.[51-53]

1.2.3. Capillary electrophoresis mass spectrometry

For years, CE has been considered a newcomer to the analytical toolbox in metabolomics.

CE in the simplest mode, capillary zone electrophoresis (CZE), has mainly been used for CE-MS analysis of metabolites, due to the simplicity of the running buffer (background electrolyte (BGE)), and the lack of surfactant or other additives necessary in other modes of separation. In CZE, charged molecules are separated based upon their differential electrophoretic mobility, while neutral molecules migrate through the capillary using the EOF without separation. Simultaneous separation of charged and neutral metabolites can be achieved using other CE modes (e.g. micellar electrokinetic chromatography (MEKC) or capillary electrochromatography (CEC)). The separation is based on the combined effect of the analytes electrophoretic mobility as well as the analytes partitioning between the mobile phase and a micellar phase in MEKC, or a stationary phase in CEC. Within CE systems, changing the polarity, coating the capillaries or modifying the BGE can modify selectivity.[54]

The main advantages of CE compared with other analytical techniques include: its small sample requirement (a few nanolitres), which is particularly

well-suited when analyzing valuable biological fluids (e.g. CSF); minor sample treatment, just enough to avoid capillary clogging, because capillaries are rinsed after each run, and apparently no irreversible retention is produced; ability to separate compounds in aqueous and non-aqueous media; capability of providing information complementary to mass, because analytes migration is based on mass to charge ratio; high efficiency which means high resolution, due to the plug-flow profile generated by electroosmotic flow (EOF); multiple modes can be applied on the same sample to generate a broader picture; the running costs are low, due to very low organic solvent consumption, the small amount of reagents and the use of inexpensive fused-silica capillaries.

The main drawbacks are: lack of robustness, shifts in retention time that complicate serial analysis; and poor concentration sensitivity, related to the relatively small on-capillary injection volumes, which is often cited as a disadvantage of CE when fitted with absorbance-related detector, which is probably the most widely used detection mode with CE because of the simplicity of the on-line configuration. However, its sensitivity, directly related to the optical path length afforded by the internal diameter of the capillary is low, and this remains the major bottleneck of this technique. Even so, small volumes do not pose a significant problem with MS for detection. Also, there several sample preconcentration techniques available to increase the sensitivity for the low-abundance endogenous metabolites.[29,41]

The on-line combination of CE and MS is an attractive option, and presents some major benefits: it enhances sensitivity and enables the determination of co-migrating compounds with different mass to charge ratios (m/z). MS provides a high potential for the identification and confirmation of components in complex mixtures and potentially gives some information concerning the structure of the separated compounds. Therefore, CE-MS coupling provides a powerful combination for performing rapid, efficient and sensitive analysis.

Almost all types of mass analyzers, such as quadruple, IT, TOF and Fourier transform ion cyclotron, can be coupled to CE, generally using ESI as the ionization source. Because of their relatively low cost, single quadrupoles remain though, mainly as mass-selective detectors, but offer limited value if structural information on the analytes is required. ITs are employed more extensively enabling tandem MS to be performed without the need for multiple analyzers. However, the typically narrow peaks resulting from CE separations, in addition to sample complexity require high mass accuracy and high resolution to be able to resolve closely migrating components with similar nominal masses. For that reason TOF are used in most of the applications. TOF technology presents numerous advantages over other analyzers, such as high mass resolution, high mass accuracy, theoretically unlimited mass range and relatively low cost. Moreover, TOF/MS is ideal for pulsed or spatially confined ionization, and a complete mass spectrum for each ionization event can be obtained, as well as spectra from extremely small sample amounts.[41]

ESI is one of the most commonly used atmospheric pressure interfacing techniques for coupling CE (as well as LC) to MS. An alternative ionization techniques include sonic spray ionization (SSI), thermo-spray ionization (TSI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photo-ionization (APPI), matrix-assisted laser desorption ionization (MALDI) and continuous-flow fast atom bombardment (CF-FAB).[55]

ESI enables the direct transfer of molecules from the liquid to the gas phase. One characteristic of ESI is the formation of highly charged ions without fragmentation, lowering the m/z values to a range easily measured by different types of mass analyzers. Interfacing CE with MS via an ESI source can roughly performed in two different ways, with or without an additional liquid for completing the CE electrical circuit for analyte separation, while simultaneously providing an electrical potential to the spray tip. The first approach, known as the sheath-flow interface, is the most common one due to its robustness and ease of implementation, while the second one (the sheathless approach) should feature a higher sensitivity, but technical difficulties in configuring them have largely precluded their routine use. ESI is particularly suited for the analysis of polar compounds and is often preferred for profiling ‘‘unknown’’ metabolites, since this ‘‘soft’’ ionization approach forms intact molecular ions and aids initial identification. But is rather inefficient for non-polar ones. Moreover, it suffers from low tolerance of salts and susceptibility to matrix effects; thus, good resolution of sample components is important for limiting competing ionizations. [29,39,41,55]

Applications of CE-MALDI-MS mainly concern analysis of peptides and (biological) macromolecules; it became the method of choice in the field of proteomics. In a typical MALDI experiment the sample is diluted and co-crystallized on a probe with a suitable matrix. Thereafter, the probe is transferred into a high vacuum ionization chamber, and irradiating the sample spot with a laser beam for analyte desorption provides the required energy input. The matrix usually consists of one or more organic acids that co-crystallize well with the analytes which absorb strongly at the wavelength of the laser. Due to the extended mass range required for detection of singly charged macromolecules, and the pulsed nature of ion generation, MALDI is predominantly combined with TOF mass analyzers. CE MALDI-MS is most often employed through collection of discrete CE fractions. An obvious drawback of this approach is the accumulation of CE effluent over discrete time intervals, which reduces the resolution achieved by CE.[57]

In APCI, the mobile phase containing eluting analyte is heated to relatively high temperatures, sprayed with high flow rates of nitrogen and the entire aerosol cloud is subjected to a corona discharge that creates ions. APPI has been shown to be relatively non-discriminate of non-polar compounds, and reasonably tolerant of matrix additives. The APPI interface uses a photo-ionization lamp and a dopant (a photo-ionizable molecule, e.g., acetone or toluene) to form dopant radical cations producing protonated eluent molecules,

followed by a proton-transfer reaction with the analyte. One main strength of the gas-phase ionization techniques, as compared with the spray ionization techniques, is their excellent compatibility with nonvolatile BGEs and sodium dodecyl sulphate (SDS), which is underlined by the feasibility of directly coupling MEKC with APPI (or APCI)-MS, without causing background signals or ion suppression. Nevertheless, the limited sensitivity that has been obtained with the current APCI and APPI source designs still prevents their widespread use as full complements to ESI and MALDI.[30,39,55,56]

Obviously the CE–MS coupling offers the structural identification capabilities of both mass spectrometer and migration time relationship to structure. Nevertheless, buffer systems and, therefore, separation modes have the typical constrains of MS, which are restricted to volatile buffers such as acetate and formiate, which often do not provide the same separation performance as standard buffers for CE with UV detection, such as phosphate and borate. In addition, their concentrations must be kept low so as not to impair ion production, and this generally results in lower efficiencies.[29]

The success of a CE-ESI-MS analysis depends not only on the characteristics of the analyte and its concentration, but also on the instrumental parameters. Besides the experimental conditions (e.g. buffer type and concentration, pH, use of organic modifiers, voltage and injection time), a stable ESI operation can be achieved by optimizing several interface parameters, including the effluent liquid (e.g. flow rate, composition) and the source itself (e.g. capillary tip position, applied voltage).

There is a growing interest in the analysis of biomolecules, natural compounds and pharmaceutics[39]. CE is particularly appropriate and offers many benefits: analysis times are reduced, minute amounts of sample are needed, and high efficiencies are obtained. In protein analysis for instance, CE allows the analysis of intact forms, in contrast to LC analysis, which usually involves an upstream digestion of the protein sample. Furthermore, CE features the possibility of analyzing them without causing conformational changes due to organic modifiers and/or a stationary phase.

CZE-ESI-MS has been used for the target profiling of amino acids in urine[57] and in CBF using non-covalently coated capillary[58]. It has been successively used to establish polypeptide patterns in dialysis fluids[59], where urine samples were desalted and the polypeptides were pre-concentrated using a C_2 column prior to the analysis. More than 600 polypeptides were found in one individual sample.

Direct CE-MS/MS has been used for the analysis of urine samples from patients with different metabolic disorders, including galactosemia, neuroblastoma, Zellweger syndrome, propionic acidemia and alcaptonuria[60]. These studies show that CE-ESI-TOF-MS can be a powerful tool in clinical metabolomic studies.

Also, CE-ESI-MS has been shown to be a valuable tool in bacterial metabolomics[61,62]. It has been used to monitor differences between intracellular

pool of sugar nucleotides, of parent and isogenic mutants from the pathogen Campylobacter jejuni[61]. By using product ion scanning, it was possible to determine the precise nature of unexpected sugar nucleotides involved in the biosynthesis of pseudaminic acid. The authors employed sample stacking and obtained as much as 1000-fold increase in sensitivity compared to conventional CE–MS.

A large-scale metabolite analysis of Bacillus subtilis extracts for revealing significant changes in metabolites during sporulation has been demonstrated by using CE-ESI-MS[62].

A similar approach was utilized for the analysis of rice leaves, successfully detecting 88 major metabolites that are involved in energy metabolism (e.g., glycolysis, tricarboxylic acid cycle, pentose phosphate pathway, photorespiration, and amino acid biosythesis)[63].

CZE-ESI-MS was applied to identify and characterize the phenolic contents of different varieties of extra-virgin olive oil[64]. Phenolic compounds in olive oil are acknowledged to be largely responsible for their antioxidant properties, which in turn have been related to their protective effect against chronic and degenerative diseases.

Additionally, metabolomics procedures based on CE-TOF/MS could open new perspectives in the study of GMOs, in order to corroborate (or not) their substantial equivalence with their conventional counterparts.[65,66]

Matching their migration time and MS or tandem MS spectra against those of pure compounds generally identifies metabolites. However, many metabolite standards are not commercially available and metabolomics-specific mass spectral libraries are still limited at this time. Therefore, identification of hundreds or thousands of metabolites can become very time consuming. For unknown compounds, the use of high-mass accuracy analyzers (e.g., TOF) permits assignment of empirical formulae, while tandem MS enables structural identification via interpretation of their fragmentation patterns.

For quantitation with ESI–MS, it must be emphasized that most of the uncertainty and potential nonlinearity do not refer to the MS, but rather to the ESI process; thus careful attention has to be given to coupling and separation. An internal standard is the best tool to compensate for changes in ionization efficiency.[6,7,54,58]

CE–MS definitely has a place in metabolomics research. It has the potential to provide useful, informative, metabolic profiles of a biological system. Though reports up to this time are far outnumbered by GC–MS- and LC–MS-based ones, and most of these deal with targeted, rather than comprehensive, metabolite analyses, its usefulness for polar analytes has been clearly demonstrated. With the availability of APPI as MS interface, which is able to generate ions from nonpolar compounds and also extend the range of MS-compatible buffer components and additives, CE–MS will be more broadly applicable.[54]

1.3. Principal component analysis of raw data

In general metabolomic patterns (either NMR, CE-MS or HPLC-MS) for different objects form a table, which is a matrix in linear algebra terms. Those multivariate data matrices require the use of mathematical and statistical procedures, in order to efficiently extract the maximum useful information from data.

Multivariate statistical tools employed for the analysis of metabolite profiling data can be classified as unsupervised and supervised approaches. In general, unsupervised methods e.g. principal components analysis (PCA), are first employed. After that supervised methods such as discriminate analysis (DA), e.g. projection to latent structures (or partial least-squares; PLS) and orthogonal projection to latent structures (O-PLS), enhance class separation, which can prove very effective for processing large data sets. PCA is often followed by PLS-DA or O-PLS-DA, where PLS-DA is used to enhance the separation between groups of observations by rotating PCA components, in such a way that a maximum separation among classes is obtained. The basic principles behind PLS-DA are similar to that of PCA, but in PLS-DA a second piece of information is used, namely, the labeled set of class identities. O-PLS-DA, similar to PLS-DA, has improved predictive capability due to separate modeling of the structured noise.[10,67]

PCA is a way of identifying patterns in the data and expressing them in such a way that similarities and differences can be seen, reducing the dimensionality without losing too much information. It gives a simplified lower-dimensional representation of the variation that is presented in a dataset. PCA transforms the original measured variables into new uncorrelated variables called *principal components* (PC). Each principal component is a linear combination of the original measured variables. This technique affords a group of orthogonal axes that represent the directions of greatest variance in the data. The first principal component (PC1) explains the maximum variance in the data; the second (PC2) explains the maximum variance in the data that has not been explained by PC1, and so on, until the total variance is accounted for. For practical reasons, it is sufficient to retain only those components that account for a large percentage of the total variance. The linear coefficients of the inverse relation of linear combinations are called the component *loadings*, i.e. the correlation coefficients between the original variables and the principal components. The values that represent the samples in the space defined by the PCs are the component *scores*. The scores can be used as input to other multivariate techniques, instead of the original measured variables.[68]

PCA is most commonly used[69,70] in order to identify how one sample is different from another, which variables contribute most to this difference, and whether those variables contribute in the same way (i.e. are correlated) or independently (i.e. uncorrelated) from each other.

While chemometric approaches like PCA and PLS-DA, on their own, do not permit the direct identification or quantification of compounds they still allow

an unbiased (or untargeted), chemically comprehensive comparison to be made among different samples.

2. EXPERIMENTAL

2.1. Instrumental and conditions

2.1.1. Capillary electrophoresis

In the first publication, CE-UV analyses were carried out with a P/ACE 2100 (Beckman Instruments, Fullerton, CA) CE equipment with UV detection. CE instrument was controlled by a PC running the system GOLD software from Beckman. An uncoated fused-silica capillary with dimensions 47 cm of total length, 40 cm effective length, 50 μm i.d., and 375 μm o.d. was used.

Initially, a new capillary was preconditioned by rinsing with 1 M NaOH for 20 min, followed by a 10 min rinse with beginning and at the end of each day, the capillary was washed with 1.0 M NaOH and water for 5 min and BGE for 15 min. Between runs, the capillary was reconditioned with BGE for 3 min. The applied voltage was 20 kV; samples were injected hydro-dynamically (22 nL, 15 s at 0.5 psi) and detected at 200 and 280 nm.

CE-ESI-TOF-MS analyses were carried out with a P/ACE 5010 CE apparatus from Beckman Instruments coupled with an orthogonal electro-spray interface (ESI, model G1607A from Agilent Technologies, Palo Alto, CA) to a microTOF MS detector from Bruker Daltonik (Bremen, Germany). An uncoated fused-silica capillary with 50 μm i.d., 375 μm o.d., and 80 cm of total (and detection) length was used. CE washing protocol, CE conditions, and sample injection were the same as the optimized by CE-UV. The nebulizing gas and voltage were stopped during the washing procedure. Electrical contact at the electro-spray needle tip was established via a sheath liquid delivered by a 74900-00-05 Cole Parmer syringe pump (Vernon Hills, IL). The MS was operated in the 50-450 *m*/*z* range during separation. Nebulizing gas, dry gas flow rate, and dry gas temperature were varied, as well as the nature of the sheath liquid and its flow rate in order to increase the sensitivity of the detection (vide infra).

In the third publication, all experiments were performed using an Agilent CE System (Agilent Technologies, Waldbronn, Germany) with a UV-Vis diode array detector (DAD). A CE Chemstation (Agilent Technologies) was used for instrument control and data handling. The separation of phenolic compounds was performed in a fused silica capillary (Polymicro Technology, Phoenix, AZ, USA) with 50 μm i.d., 60 cm total length and 52 cm effective length. Prior to use, the capillary was rinsed with a 0.1 M NaOH solution for 5 min and with the separation buffer for 5 min. A 50 mM sodium tetraborate (pH 9.3) as a BGE was used. All measurements were carried out at 25 ºC. The diode array detection was used over the range of 190-600 nm to obtain spectral data. The applied voltage for the separation of polyphenols was 20 kV: samples were injected under 50 mbar pressure for 8 s and detected at 210 and 254 nm.

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2.1.2. Liquid chromatography

In the second and fourth publication, samples were analyzed using LC/ESI-MS/MS in negative ionization mode on an 1100 Series LC/MSD Trap-XCT (Agilent Technologies, Santa Cruz, CA, USA). The ion trap was connected to an Agilent 1100 Series HPLC instrument consisting of an autosampler, a solvent membrane degasser, a binary pump and a column thermostat. The HPLC 2D ChemStation software with a ChemStation Spectral SW module was used both for process guidance and for the processing of the results. The samples were separated on a Zorbax 300SB-C18 column (2.1x150 mm; 5 μm particle size; Agilent Technologies) with a guard column filled with the same type of sorbent.

The column was eluted at 0.3 mL/min with a linear gradient from 0.1% aqueous formic acid (solvent A) and 5% acetonitrile (solvent B) to 30% B in 40 min, followed by an increase to 90% B over 15 min. The column temperature was maintained at 35 °C and the sample injection volume was 15 mL.

The conditions of MS/MS detection: *m/z* interval, 100–1000 amu; target mass, 400 amu; number of fragmented ions, 2; maximal collection time, 100 ms; compound stability, 100%; drying gas (N_2 from generator) speed, 10 L/min; gas temperature, 350 °C; gas pressure, 30 psi; collision gas (He) pressure, $6 \cdot 10^{-6}$ mbar.

2.1.3. Spectrofotometry

In the third publication, total phenolic content of free phenolics was determined by using the Folin-Ciocalteau method. Each fraction of free phenolics (0.2 ml) was mixed with 1 ml of the Folin-Ciocalteau reagent and 0.8 ml of a saturated sodium carbonate (20%) solution. The mixture was allowed to stand at room temperature for 30 min and then the absorbance was measured at 765 nm in a Varian Cary 3C spectrophotometer (Varian analytical instruments, Harbour City, CA). The total phenolic content was expressed as microgram of gallic acid equivalent (GAE) per milliliter of solution. From this data the total phenolic contents of wheat bran, grain and flour were subsequently calculated.

The free radical scavenging activity of wheat bran extracts was determined using a 1 mM 2,2-diphenyl-1-hydrazyl (DPPH) solution. Each sample of wheat extracts at different concentrations in methanol (2 ml) was mixed with 2 ml of a methanolic solution containing 1 mM DPPH. The mixture was shaken, and then left to stand for 30 min in the dark. The absorbance was measured at 517 nm. The absorbance of the control was obtained by replacing the sample with methanol. The DPPH radical scavenging activity of the sample was calculated as follows:

DPPH radical scavenging activity (%) = [1- absorbance of sample/absorbance of control] x 100.

The EC_{50} value was determined to be an effective concentration at which the DPPH radical was scavenged by 50%. The EC_{50} value was obtained by interpolation from a linear regression analysis.

2.2. Chemicals and reagents

2.2.1. Chemicals and reagents used in CE applications

In CE-MS application, methanol and acetonitrile from Scharlau (Barcelona, Spain), chloroform from Solvents Documentation Syntheses (Peypin, France), acetone from Labscan (Dublin, Ireland), and ethanol from Prolabo (Fountenay sous Bois, France) were used as extraction solvents. Ammonium hydrogen carbonate from Fluka (Buchs, Switzerland), formic acid from Riedel-de Haën (Seelze, Germany), acetic acid from Merck (Darmstadt, Germany), ammonium hydrogen acetate from Panreac Qu´ımica S.A. (Barcelona, Spain), and 2-propanol from Scharlau, were used for the CE running buffers and sheath liquids. A water solution containing 0.1 M sodium hydroxide from Panreac Quımica S.A. and 1% SDS from Fluka was used for capillary washing before each analysis.

In CE-DAD application methanol from Fluka (Buchs, Switzerland) and diethyl ether from Sigma–Aldrich (Germany) were used for extraction. Sodium tetraborate, sodium hydroxide, sodium sulfate anhydrous, hydrochloric acid and standard phenolic acids (syringic, vanillic, ferulic, p-coumaric, caffeic, gallic and sinapic acids) were purchased from Sigma-Aldrich (Germany).

2.2.2. Chemicals and reagents used in LC-MS applications

Methanol and formic acid from Fluka (Buchs, Switzerland) were used for extraction and separation, respectively. Acetonitrile and methanol of ultra-gradient grade used in the chromatographic experiments were from Romil (Cambridge, UK). Standard oxylipins [9-HODE, 13-HODE, 9,10-dihydroxy-12-octadecenoic acid (9,10-DiHOME)] were purchased from Cayman Europe (Tallinn, Estonia).

2.2.3. Reagents used in spectrophotometric applications

Folin-Ciocalteau reagent was used for determination of total phenolic content, purchased from Sigma-Aldrich (Germany).

Antioxidative capability was assessed by the DPPH radical reaction. DPPH was purchased from Romil (Cambridge, UK).

All chemicals, used throughout in publications, were of analytical reagent grade. Deionized water was prepared by using a Milli-Q system from Millipore (Bedford, MA, USA).

2.2.4. Samples

In the first publication the investigated varieties of conventional and transgenic maize were obtained from a field assay carried out in Estacio´ n Experimental Agr´ıcola Mas Bad´ıa in Tallada d′Emporda´ (Girona, Spain) using commercial varieties. Namely, in order to skip any influence from the growing conditions, Aristis maize (wild type and its Bt transgenic variety), Tietar maize (wild type and its Bt transgenic variety), and PR33P66 maize (wild type and its Bt

transgenic variety) were grown under the same field conditions and investigated in this work. The transgenic and non-transgenic nature of all these maize samples was confirmed based on their DNA.

In the second, third, and fourth publication different varieties of wheat grains, bran and flour were obtained from the Jõgeva Plant Breeding Institute (Estonia) (all harvested in 2007-2009).

2.3. Sample preparation

A simple ultrasonic extraction procedure with a widely used solvent (and its compositional variability) was selected for maize extraction. The same extraction procedure was applied further for extraction of wheat with some distinctions followed from used instruments and specificity of successive works.

In the first publication, different solvents (water, methanol, ethanol, acetonitrile, acetone), solvent mixtures, and sample amounts were tried, to optimize the extraction, in order to obtain a fraction from maize flour highly informative in a reproducible way. The maize grains were milled to a fine powder using an ordinary grinder. Namely, 0.1, 0.5, or 2 g of maize flour were weighed and extracted using 3, 6, or 10 mL of solvent (methanol, acetonitrile, acetone, water, and mixtures of ethanol/water, methanol/water with two different ratios, 50:50 and 75:25), in an ultrasonic bath for 10 min. After sonication, samples were centrifuged for 5 min at 3000 rpm and liquid phases were filtered through a 0.45 μm filter. Liquid phases were taken to dryness in a rotatory evaporator and re-dissolved in 0.5 mL of solvent before injection into the CE system.

For comparison[71,72], a different extraction procedure was also tested, adding 3 mL of methanol/chloroform mixture (2:1) to the flour of maize. The samples were stirred, and 1 mL of chloroform and 1.2 mL of water were added. Samples were stored at 4 °C for 1 h and then centrifuged at 10000*g* for 20 min at 4 °C. The resulting upper hydro-alcoholic and lower chloroformic phases were separated. The extraction procedure was performed twice and thereafter dried in rotary evaporator and re-dissolved in 1 mL of methanol.

In the second and fourth publication, the wheat grains were milled to a fine powder using an ordinary grinder. About 2.0 g of finely ground wheat was weighed and extracted with 10 mL methanol in an ultrasonic bath for 30 min. After sonication, the samples were centrifuged for 5 min (3000 rpm) and the liquid phases were filtered through 0.45 μm filters. The liquid phases were taken to dryness in a rotary evaporator and re-dissolved in 0.5 mL methanol and injected directly into the LC system. The bran layer and flour were weighed directly as received. To obtain more hydrophilic extraction conditions, methanol/water mixture in ratio 50:50 was used as a solvent a similar extraction procedure. The analytical samples obtained were stored at -18 ˚C.

In the third publication, for extraction of free phenolics, wheat grains were milled to a fine powder by using an ordinary grinder. The bran layer and flour were used as received. Three replicates (2 g each) of wheat bran layer, flour and

grain were individually mixed and sonicated with methanol for 30 min. After sonication, the samples were centrifuged for 10 min at 2500 g. The extraction procedure was repeated twice, the supernatants were pooled and evaporated to dryness and redissolved in 0.5 mL of methanol.

After the methanol extraction the residue was hydrolyzed with sodium hydroxide. 20 mL of a 2 M NaOH were mixed with 1 g of wheat bran layer, flour and grain residue. The mixture was mixed for 4 h and the pH was adjusted to two with a 6 M HCl. Diethyl ether (100 mL) was added to the mixture, the container was inverted 15 times and then centrifuged at 1000 g for 10 min. The supernatant was removed and the process was repeated with 75 mL of diethyl ether. The supernatants were pooled, dried with anhydrous Na_2SO_4, evaporated to dryness and redissolved in 0.5 mL of methanol.

2.4. Data processing

In the first publication, to detect statistically significant peak area differences, in the electropherograms of different maize varieties, a Student's *t* value was initially calculated as follows[73]:

$$t_{\exp} = \frac{\left|A_i^W - A_i^{GMO}\right|}{\sqrt{\left(s_i^W\right)^2 + \left(s_i^{GMO}\right)^2}}\sqrt{n}$$

Here A_i^W is *i*th peak area for wild variety and s_i^W is its standard deviation. Index GMO denotes peak area and standard deviation for genetically modified variety and *n* is number of measurements. Since $n = 3$ in this work, the number of degrees of freedom is $2 \times 3 - 2 = 4$ and critical two-tailed Student's *t* value for 99% confidence level is $t_{crt} = 4.6$.

For PCA, the electropherograms and chromatograms of cereal varieties were transformed to a table (a matrix) of metabolites peak areas either intensities. In this table a row corresponds to a certain variety, and a column to a metabolite (represented via a corresponding chromatographic peak area either intensity). If we denote this matrix as, *D*, the PCA procedure decomposes the matrix *D* as follows: $D=ST^T$ (here superscript means transpose). Assuming that dimension of *D* is *nxm*, where *n* is a number of varieties under study and *m* is a number of measured peaks, the dimension of a scores matrix, *S*, is *nxp* and the dimension of the loadings matrix *T,* is *mxp* where $p<<n$. Plotting first row of *S* versus its second row a PCA plot is obtained where each point represents a variety. Moreover, if first two components of *T* are overlaid onto the scores plot as vectors, the directions of these vectors explain scatter and clustering of the varieties that are plotted on the scores plot.

In the first, second and fourth publication, PCA was performed to differentiate the varieties of maize and wheat by applying the peak areas either intensities of selected marker compounds obtained from CE or HPLC analysis.

PCA was carried out using a Matlab (Mathworks, Natick MA) environment using a standard singular value decomposition procedure. For CE and HPLC data processing, peak areas either heights were replaced by their logarithms and mean-centered.

3. RESULTS AND DISCUSSION

3.1. Selection case studies of interest

"Case studies of interest" i.e chosen cereals of study were selected considering their novelness, latest issues in food science, potential usefulness of informative outcome, and which were the most relevant for the research of the departments participated in Publications.

3.2. Metabolites in maize varieties (Publication I)

3.2.1. Optimization of analytical conditions

The extraction procedure was optimized in order to obtain a fraction from maize flour highly informative (i.e. higher number of peaks, assuming this means a higher number of metabolites extracted) in a reproducible way. The best results were obtained using 2 g of sample in 10 mL of methanol/ water (50:50) with ultrasounds for 10 min. The next step was to optimize the CE separation, taking into account that BGE(s) has to be compatible with the subsequent ESI-MS analysis and able to provide a fast separation of metabolites with high resolution and sensitivity. Sixteen BGE at different pH were tested, the pH of the BGEs varied from 1.9 to 8.24, and other experimental CE conditions (separation voltage, temperature, and injection time) were also evaluated in order to improve the separation. As a results of this step, two BGSs were selected: 2.5% formic acid at pH 1.9, and a second composed of 25 mM NH_4HCO_3 in 10% methanol at pH 7.82. The injection volume and the voltage were optimum at 22 nL s and 20 kV, respectively, and the temperature was set at 30 ºC. Under these conditions, good separations were obtained for both BGEs with a relatively high number of peaks and short times (less than 10 min, in both cases). This result is especially interesting considering the longer capillaries usually required for coupling CE and MS instruments.

In optimization of ESI-TOF-MS parameters, it was systematically observed that the number of compounds and sensitivity provided by the acidic BGE were in all cases better. Finally, a 5% formic acid, at the same pH other than the optimum (pH 1.9) was used since it was observed to favor slightly the resolution without increasing in excess the analysis time. The optimum conditions were 2-propanol/water (50:50) as sheath liquid flowing at 0.24 mL/h together with 0.4 bar N_2 as nebulizing gas and dry gas at 180 ^{o}C and 4 L/min. Other TOF settings were as follows: 50 μs for the transfer time and 5 μs as prepulse storage. The MS scan range was fixed in a range that allowed detecting as many metabolites as possible without reducing sensitivity; namely, the scan range from 50 to 450 *m*/*z* was selected.

The reproducibility of the method was evaluated by measuring the migration time and area of three selected peaks, injecting in triplicate three samples obtained under the same extraction conditions. The RSDn=9 values were lower than 1.5% for the migration time and lower than 12% for the peak areas.

3.2.2. Identification of metabolites

The six maize samples were analyzed. An example of the CE-TOF-MS extracted ion electropherogram obtained for two of the samples (PR33P66, PR33P66 Bt) is shown in Figure 2. As can be seen, 27 metabolites were easily found and tentatively identified as indicated in Table 1 (Appendix I). Tentative identification was carried out based on the highly accurate mass determination provided by CE-TOF-MS, which allowed for the generation of a more probable molecular formula for each metabolite. This molecular formula was then introduced in, e.g. Kegg, Chemspider, or PubChem databases to obtain the metabolite identification. These assignments were corroborated using isotopic pattern simulations. Moreover, the expected electrophoretic mobility of each compound at the separation pH was also used to further corroborate the assignments. Thus, at the separation pH of 1.9 used for the CE-TOF-MS electropherograms of Figure 2, all the amino groups will be positively charged, while the acid groups will be practically neutral or scarcely ionized, giving a global positive charge to all the metabolites that share these two groups in their molecule (for example, amino acids), allowing their migration toward the MS detector under the separation voltage applied.

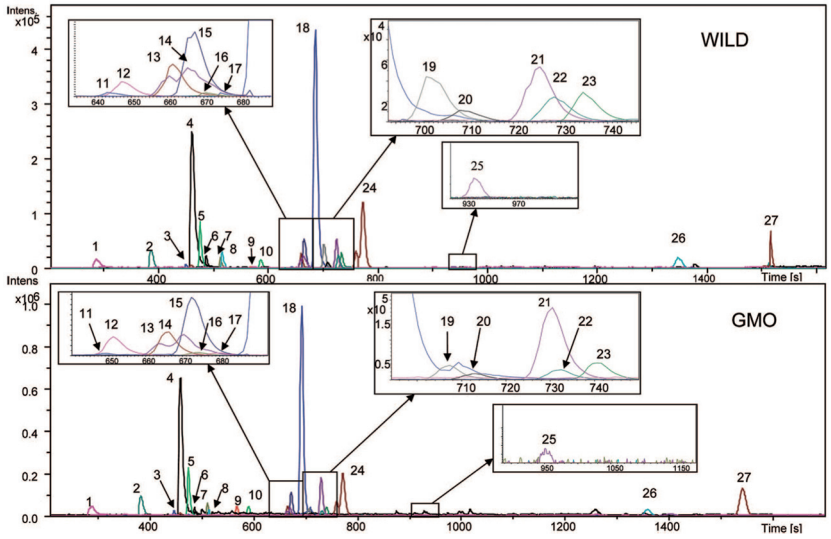

Figure 2. CE-TOF-MS extracted ion electropherograms of the 27 metabolites detected in PR33P66 and PR33P66 Bt maize. Tentative identification of 27 metabolites are shown in Appendix I. Experimental conditions: BGE composed of 5% formic acid at pH 1.90, total length of the capillary 80.0 cm; 50 µm i.d.; applied voltage 20 kV; volume injected 22 nL; temperature 30 °C. Sheath liquid 2-propanol/water (50:50), at 0.24 mL/h, nebulizer pressure was 0.4 bar and dry gas conditions 4 L/min N_2 at 180 °C. MS scan range from 50 to 450 *m*/*z*.

Thus, as expected, compounds with a higher number of amino groups in their structure such as 3,5-diaminocaproate, subaphyllin, or arginine are observed to migrate first. A second group of metabolites migrates subsequently formed by different amino acids (GABA, carnitine, alanine, serine, valine, leucine, threonine, proline), including other metabolites such as trigonelline, a product from the metabolism of vitamin B3,[74] or amino acid derivatives such as homoproline or small peptides like β-alanyl-L-arginine, with lower charge/mass ratio and, therefore, lower mobility. The next migrating group is formed by less positive amino acids such as tyrosine, glutamic acid, and aspartic acid as well as other acidic metabolites such as 7-keto-8-pelargonic acid and stachydrin, with a higher ratio of oxygen in their molecules. This leads to higher migration times, due to the charge compensation induced by the carboxylic groups. Finally, a group of alkaloids (as lunarine or graveoline) together with a nucleoside and a polyphenol, all with higher oxygen content in their structure, are detected at the end of the separation. This behavior is explained considering that these compounds, although still bearing some positive electrical charge, show a very low positive character due to the higher amount of negatively ionizable groups in the molecule that partially compensate their positive charge.

In Table 1 (Appendix I), a comparison between the relative molecular mass values found for metabolites in the transgenic ($M_{r\ GMO}$) and the wild maize ($M_{r\ wild}$) is shown, together with a comparison with the theoretically expected values, observing in all cases a good agreement. Moreover, the ratio between the peak area of each compound determined in the transgenic and wild maize is also included (A_{GMO}/A_{wild}). This ratio could indicate possible detected metabolites that are under- or over-expressed in genetically modified maize varieties. Using the Student's *t*-test, two compounds (peaks 9 and 21) were found to have statistically significant differences in peak areas in all varieties (i.e $t_{exp} > 4.6$). Namely, metabolite 9 with a molecular weight of 161.106, identified as L-carnitine, is clearly over-expressed in all the GMO samples. Thus, L-carnitine could be considered as a possible biomarker for the detection of transgenic samples. L-carnitine is involved in the fatty acid metabolism, enhancing the transport of fatty acids inside the mitochondria for their oxidation, but also in the glucose metabolism favoring the glycogen storage.[75-77] Besides, compound 21, with a molecular weight of 143.094 and identified as stachydrine, also called L-proline-betaine, could also be used as a potential biomarker, since it is over-expressed in all the three GMO samples. Although a larger number of samples need to be analyzed in order to confirm this point, it is interesting to remark that the statistically significant difference was observed in all the investigated samples (including the other two couples of maize, Aristis/Aristis Bt and Tietar/Tietar Bt) for only 2 compounds out of the 27 metabolites identified.

Thus, the other two compounds differing more than statistically expected, are metabolite 19 (identified as glutamic acid) in variety Aristis and 13 (identified as homoproline) in both varieties PR33P66 and Aristis. However, since the

statistically significant difference for metabolites 13 and 19 is not systematically observed for all the samples, their difference can more likely be explained through natural variability.

3.2.3. Transgenic vs. conventional maize: PCA results

To confirm the conclusions obtained from the Student's t-test, the data as a whole were next subjected to PCA. The data matrix has dimensionality 18 x 7 (seven CE-MS peak areas chosen from electropherograms of six varieties). In Figure 3, CE-MS data are represented in two first principal component coordinates that account 70% of data variability.

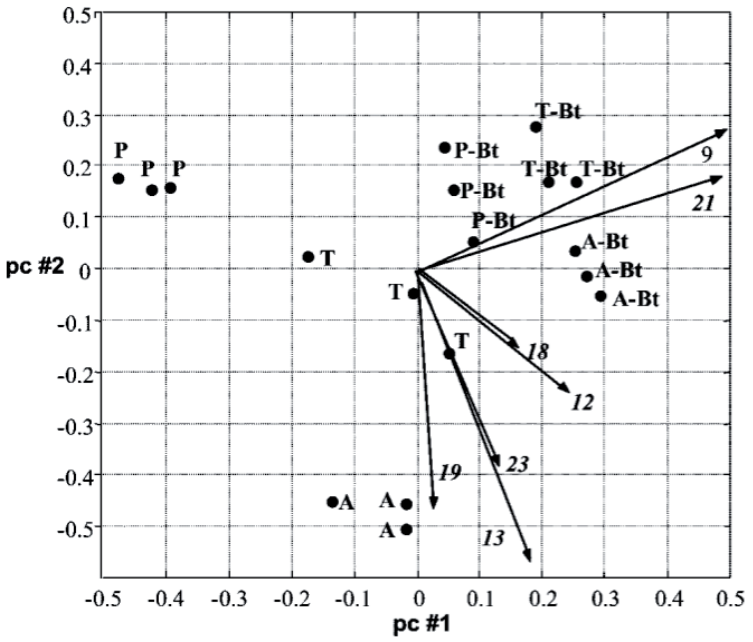

Figure 3. Representation of the CE-MS electropherograms in the first principal component coordinates (accounting of the 70% variability). "A" stands for conventional Aristis maize. "P" for conventional PR33P66 maize, and "T" for conventional Tietar maize, while the terms, "A-Bt", "P-Bt", and "T-Bt" correspond to their respective transgenic varieties. Vectors are loadings and numbers 9, 12, 13, 18, 19, 21 and 23 refer to peaks in CE-MS electropherograms.

Each point represents one particular electropherogram. As can be seen in Figure 3, conventional (A, P, and T points) and transgenic (A- Bt, P-Bt, and T-Bt points) varieties are nicely separated using this approach. To find out what feature (peak) is responsible for the separation of samples (i.e., sorting), loading vectors located in columns of *p* matrix are also represented in Figure 3. It is evident from Figure 3 that vectors corresponding to peaks 9 and 21 are the main

ones responsible for the separation of wild and transgenic varieties. Therefore, this PCA confirms the result of Student's *t*-test.

3.3. Oxylipins in wheat varieties (Publication II)

3.3.1. Identification of metabolites

The eight wheat (*Triticum aestivum*) samples were analyzed. Extraction procedure is described at section 2.3. In this study, the extraction conditions were selected on the basis of the polarity of oxylipins. Using methanol as an extraction solvent allowed us to perform the extraction in a reproducible way and to guarantee good stability of the extracts. As the primary goal of this work was to provide qualitative information about oxylipins in wheat, the extraction was performed only once.

LC-ESI-MS analysis of wheat extracts revealed the presence of different metabolites. An example of the LC-MS extracted ion chromatograms obtained for two of the samples (Azurite, Anthus) are shown in Figure 4. As can be seen, a lot of metabolites were easily found and oxylipins, as an interest group, were possible identify. All the oxylipins identified (Fig. 4, peaks 1–10) are various (per)oxidation products of linoleic (*cis*,*cis*-9,12-octadecadienoic) acid, most of them are characterized by the MS^2/MS daughter ion $–OOC(CH_2)_7CH\text{-}OH$ with *m/z* = 171 amu, containing a carboxyl and an OH-group. Neutral loss of 100 amu (*e.g.* 329–229; 295–195; 311–211) corresponds to the loss of the end-group $HO\text{-}CH{=}CH(CH_2)_3CH_3$ from an oxylipin molecule. Between these groups, for example, the fatty acid with $[M–H]^-$ = 329 has a moiety $–CH_2CH(OH)\text{-}CH_2$- that corresponds to the neutral loss of 58 amu (229–171). The molecular structures of the compounds were verified either by comparison of the daughter ion spectra with the respective spectra of standard fatty acids (9-HODE, 13-HODE, 9,10-DiHOME), or with the respective spectra from the literature.[78-82] In some cases, chromatographically un-resolvable mixtures of positional isomers as well as stereoisomers of an oxylipin were formed. For example, one of the highest peaks in the base peak chromatogram is characterized by a pseudo-molecular ion $[M–H]^-$ = 295, representing a mixture of two HODE (9- HODE and 13-HODE) (Fig. 4, peak 9). Fatty acid negative ions with $[M–H]^-$ = 293, 297, 309, 313, 327, 331, all possessing the characteristic negative daughter ion 171, were also discovered. Further investigations should clarify the complicated oxidation scheme of PUFA producing all these oxylipins.

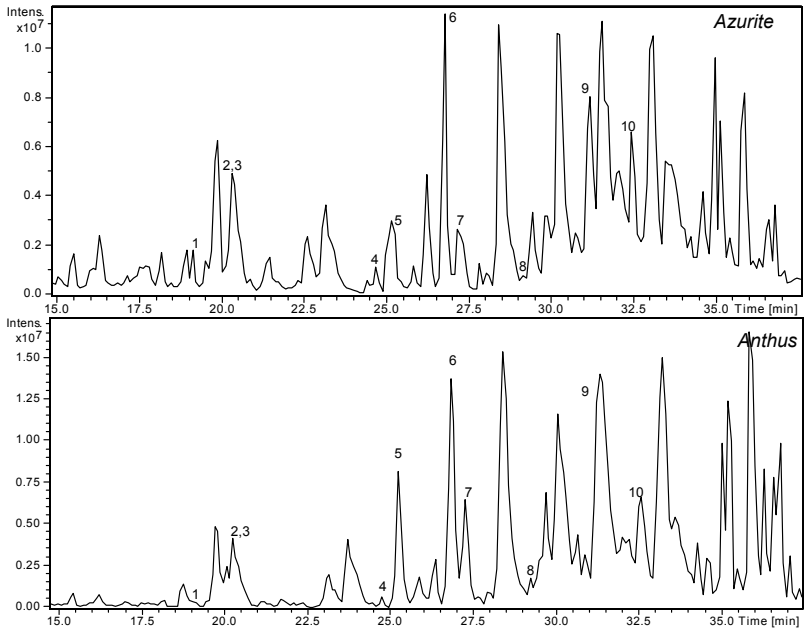

Figure 4. LC/ESI-MS/MS base peak chromatograms of the methanol extracts of Azurite and Anthus, a spring and a winter wheat variety, respectively. Peak numbers indicate the tentative oxylipins detected: (1) mixture of 9-oxo-12,13-dihydroxy-10-octadecenoic (9-oxo- DHODE) and 13-oxo-9,10-dihydroxy-11-octadecenoic (13-oxo-DHODE) acids ($[M–H]^-$ = 327); (2) 9,10,18-trihydroxy-octadecanoic acid (9,10,18-THODE) ($[M–H]^-$ = 331); (3) 9,12,13-trihydroxy-10-octadecenoic (9,12,13-THODE) acid ($[M–H]^-$ = 329); (4) 9-hydroperoxy- 10,12,15-octadecatrienoic acid ($[M–H]^-$ = 309); (5) mixture of 13-hydroxy-9-oxo-10-octadecenoic (9-oxo-13-HODE) and 13-oxo-9- hydroxy-10-octadecenoic (13-oxo-9-HODE) acids ($[M–H]^-$ = 311); (6) 12,13-dihydroxy-9-octadecenoic acid (12, 13-DiHOME, iso-LTX-diol ($[M–H]^-$ = 313); (7) 9,10-dihydroxy-12-octadecenoic acid (9,10-DiHOME, LTX-diol) ($[M–H]^-$ = 313); (8) 15,16-epoxy-12,15-octadidecenoic acid (15,16-EODE) ($[M–H]^-$ = 293); (9) mixture of 13-hydroxy-9,11-octadecadienoic (13-HODE) and 9-hydroxy-10,12-octadecadienoic (9- HODE) acids ($[M–H]^-$ = 295); (10) 9,10-epoxyoctadecanoic acid (9,10-EODA) ($[M–H]^-$ = 297).

The health deteriorating effect of oxidized fats has been known for decades[83], but mostly it has not been associated with concrete oxidation products. One of the few exceptions is 9,10-DiHOME ($[M–H]^-$ = 313; peak 7 in Fig. 4), known also as leukotoxin diol (LTX-diol) and its isomer 12,13-dihydroxy-9-octadecenoic acid or isoleukotoxin-diol (iso-LTX- diol; peak 6 in Fig. 4), which were discovered by us in wheat grains. The structure of these compounds was proved by comparison of their MS^2/MS daughter ion spectra with both the

spectra reported in the literature[79] and with the fragmentation spectra of the respective commercial standards. All these fragmentation spectra had a very good congruence.

The acute toxicity of the endogenous LTX-diols is well characterized[84-85]. Furthermore, LTX-diols exerted also mitogenic activity and stimulated human breast cancer cell proliferation *in vitro*[86]. It was recently found that exogenous LTX-diols act additively to disrupt the endocrine function in female rats[87].

Whether the LTX-diols in wheat grains have any real toxic consequence for human organisms, especially after baking, needs further investigations.

3.3.2. Spring vs. winter wheat: PCA results

In Figure 5, LC-MS/MS data are represented in two first principal component coordinates. Each point represents one particular chromatogram. As can be seen in Figure 5, the wheat varieties Ada, Manu, Picolo and Bjorke are nicely separated using this approach. To find out what feature (*m/z*) is responsible for the separation of samples, loading vectors located at columns of matrix *p* are introduced and represented in Figure 5. It is evident that vectors corresponding to the *m/z* values 297, 311 and 329 are the main responsible ones for the separation of the above-mentioned wheat varieties. Also, from Figure 5, spring and winter wheat varieties occupating two standalone areas on the PCA plot can be identified. An area of striated rectangle in Figure 5 denotes the spring wheat varieties. Those varieties that belong to either the spring or the winter group have more group-specific features and are bunched together with one exception: Anthus. Still, more grain samples of different varieties must be analyzed to confirm this point.

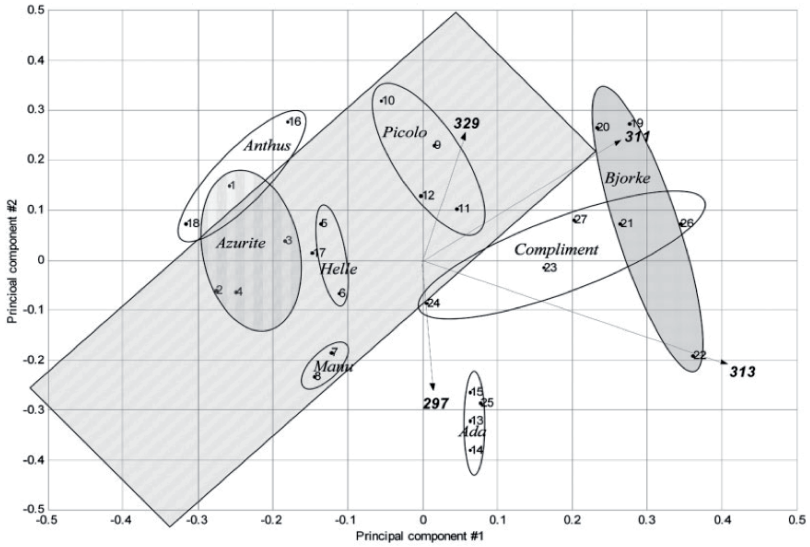

Figure 5. Representation of the LC-MS/MS chromatograms in the first principal component coordinates. Each point represents one particular chromatogram and groups encircled by ovals represent one sample (those groups illustrate measurement reproducibility). Vectors are loadings and numbers 297, 311, 313 and 329 refer to m/z-values that primarily are responsible for the scattering. A striated rectangle denotes to the spring wheat varieties.

3.4. Phenolic compounds and antioxidativity of wheat varieties (Publication III)

3.4.1. Phenolic compounds and total phenolic content

Fifteen different wheat (*Triticum aestivum*) varieties were investigated. The phenolic contents were determined by the Folin-Ciocalteau assay and expressed as microgram of gallic acid equivalent (GAE) per milliliter of solution. It was found that the bran layers have the highest content of total phenolics, it confirms the well known fact that phenolic compounds are concentrated in the bran and germ fractions of wheat which are removed during the milling of wheat into white flour. A comparison of the total phenolic contents of spring and winter wheat varieties, a later seemed to be more stable. The content of total phenolics of conventional spring varieties differed a lot, and therefore, may be assumed to be more variety-specific. Our results are highly comparable with literature[88,89], although the use of different standards for the measurements makes the respective comparison quite difficult.

Another outcome showed the total phenolic content of the spring wheat varieties grown in organic conditions is a little higher than that of the wheat grown in conventional conditions. It may be assumed that growing conditions

have a certain effect on the biosynthesis and accumulation of phenolic compounds.

From literature[90,91] it is known that the phenolic acids in wheat grains are present mostly in the bound form with other grain components such as starch, cellulose, β-glucan and pentosane. Insoluble bound phenolics may be released by the base, acid or enzymatic treatment of samples prior to extraction. In the present work, the alkaline hydrolysis was performed before extracting these compounds with diethyl ether.

To determine the concentration of each individual phenolic acid, CE was used for separation and each fraction was subsequently quantified by UV adsorption. As shown in Figure 6, six phenolic acids were separated and identified. This method is well reproducible and provides good separation in terms of migration time and resolution.

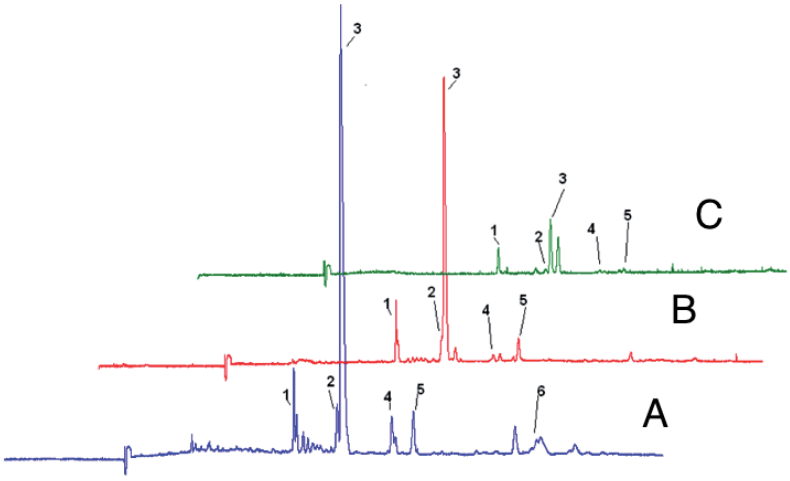

Figure 6. The electropherograms of bran (A), whole grain (B) and flour (C) extracts of the spring wheat Helle. Separation buffer: 50 mM sodium tetraborate (pH 9.3). The phenolic acids separated: 1 – sinapic acid, 2- syringic acid, 3 – ferulic acid, 4 – *p*-coumaric acid, 5 – vanillic acid, 6 - caffeic acid.

A dominant phenolic acid identified was ferulic acid, followed by sinapic, syringic, vanillic and *p*-coumaric acids. The caffeic acid was present only in the spring variety. The content of ferulic and sinapic acids of the winter variety was two times higher than that of the spring variety. Ferulic acid comprise of 48% and 60% among total phenolic acids in spring and winter wheat bran, respectively.

The total content of bound phenolic acids of the winter wheat variety is higher than that of the spring variety. It may be assumed that in the case of winter wheat the environmental conditions in the growing phase are more stressful for the plant. This is in agreement with the well known fact that

phenolic compounds, as secondary metabolites, are synthesized by plants during growing phase more in response to stress than in normal conditions.

3.4.2. Antioxidant activity

Phenolic compounds have a potent antioxidant activity; their total phenolic content has been found to be significantly associated with different measure of antioxidant activity[89] including DPPH scavenging capacity[92]. It can be seen from Figure 7 that the extracts with a higher content of phenolics possess a higher antioxidant activity (lower value of EC_{50}). The difference in antioxidant activity between different wheat varieties may be due to the different composition of the phenolic compounds present.

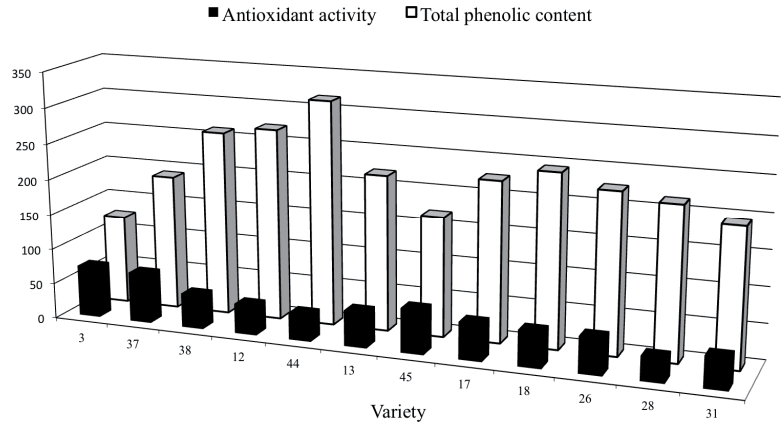

Figure 7. The total phenolic content (mg GAE /100g) and antioxidant activity EC_{50} of the bran layer of selected wheat varieties.

3.5. Metabolites in wheat varieties (Publication IV)

Thirteen different wheat *(Triticum aestivum)* varieties were analyzed. Twenty-three major metabolites were detected in wheat grain extracts, and almost fifty in the bran extracts. In this publication, a contrary approach to the previous publications was chosen. Compounds that were responsible for scattering in PCA results were interest groups for identification.

3.5.1. Spring, winter and organic wheat: PCA results

In Figure 8, the LC-MS/MS data are represented in two first principal component coordinates. Each point represents a peak height of a particular m/z value. As it can be seen in Figure 8, this approach enabled a nice separation and grouping of the investigated varieties. It is evident, that vectors, corresponding to the m/z values 341 and 452, are the main responsible ones for the separation. Also, in Figure 8, organic, spring and winter varieties as three standalone groups

can be distinguished. Those varieties that belong to spring, winter, or organic group have more common features and are bunching together, with some exception: Anthus (item 6) and Björk (item 7). Anthus was also characterized as an exception in Publication II. Still, more grain samples of different varieties must be analyzed to confirm this point.

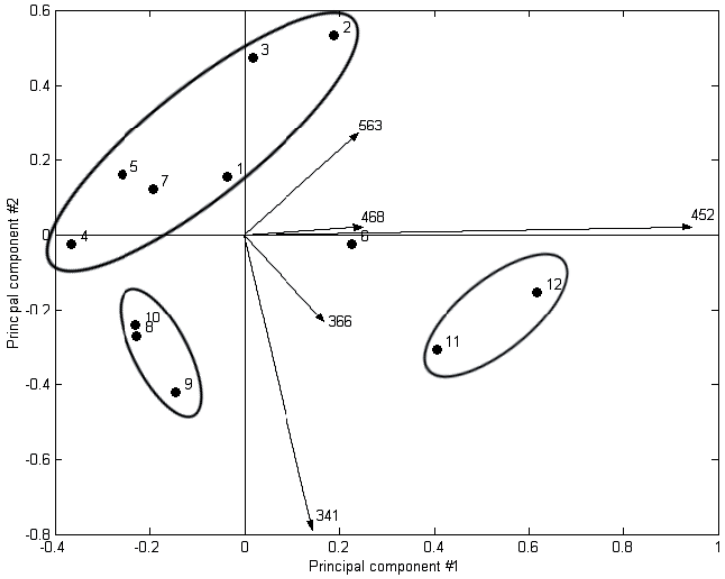

Figure 8. Representation of the LC-MS/MS base peak chromatograms of the methanol extracts of 12 wheat varieties in the first principal component coordinates (accounting for the 71% variability). Items 1-5 refers to the spring, items 6-10 to the winter and 11-12 to the organic varieties. Each point represents one particular chromatogram. Vectors are loadings and numbers 341, 366, 563, 468 and 452 refer to m/z values that primarily are responsible for the scattering.

3.5.2. Flour and bran portions and the whole grains: PCA results

Flour and the bran portions of the grain and the whole grains of the same varieties were separately analyzed. In Figure 9 chromatograms of different fractions of different varieties (Spring-Vinjett (item 4), organic-Vinjett (item 13) and winter-Bjorke (item 7)) are represented in PCA coordinates. It can be seen that vectors, corresponding to the m/z values 341 and 563, are mainly responsible for the separation. It can also be observed from Figure 9 that the bran fractions of the investigated varieties are significantly distinct from each other (items 4B, 7B and 13B). It may be assumed that differences between the bran fractions are strongly dependent on variety. Another observation that can be made; the organic variety may show characteristic behavior. It could be

explained by the organic growing conditions. Nevertheless, substantially more samples of different varieties must be analyzed to confirm the last finding.

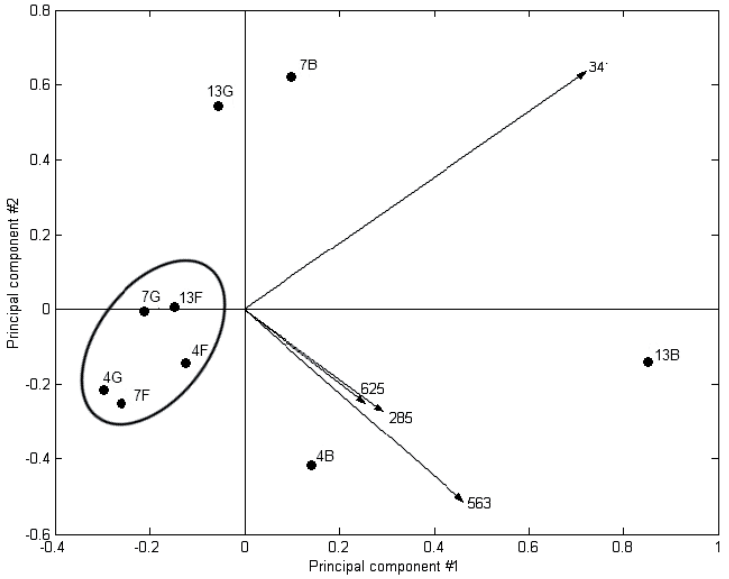

Figure 9. Representation of LC-MS/MS base peak chromatograms of the methanol extracts of grains (G), bran (B) and flour (F) of three varieties (spring-Vinjett (item 4), winter-Bjorke (item 7) and organic-Vinjett (item 13)) in the first principal component coordinates (accounting for the 67% variability). Each point represents one particular chromatogram. Vectors are loadings and numbers 341, 563, 285 and 625 refer to m/z values that primarily are responsible for the scattering.

3.5.3. Identification of selected key metabolites

In the Figure 10 is represented the sample base peak chromatogram of the grains of winter wheat variety Anthus. Table 2 (Appendix II) summarizes the characteristics of selected peaks obtained from LC-MS/MS analysis. The tentative identification was performed by interpretation of the MS/MS fragmentation patterns of corresponding analytes, their accordance with literature data and chromatographic behavior.

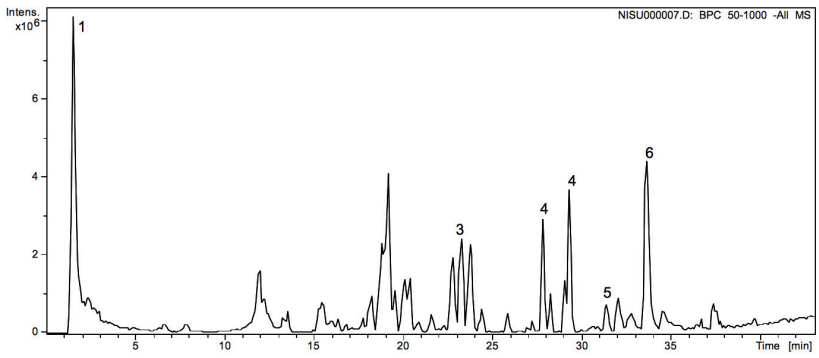

Figure 10. LC/ESI-MS/MS base peak chromatogram of the methanol extract of Anthus (whole grain), a winter wheat variety. Peak numbers indicate tentative polyphenols, primarily responsible for the scattering in PCA. 1 – unspecified disahharide; 3 - hexose-hexose-N-acetyl; 4 - apigenin-6-C-pentoside-8-C-hexoside; 5 – unknown; 6 – putatively rhamnoside.

Peak 1. A molecular ion at m/z 341 had CNL of 162 Da, which corresponds to the loss of a hexose moiety (glucose, galactose, or fructose) linked to the rest of the molecule by an O-glycosidic bond. The ions with m/z 179 and 161 indicate scission of the glycoside bond to form the complementary monohexose molecule. CNLs of H_2O and CH_2O form the other product ions, with m/z fragments 143, 119, 131 and 101, and which were present in the product ion mass spectrum from each of hexoses. Identified disaccharide could be sucrose or maltose. Under peak no 1, lower extracted ion peak of a molecular ion with m/z 503 was found. Its MS/MS fragmentation ions at m/z 377, 323, 341 and 179 refer to the tri-glucoside, but it had too low peak intensity for further PCA. Detected tri-glucoside can be raffinose, which is found in wheat as well. The content of m/z 341 in the organic and winter samples is almost twofold in comparison with the spring varieties (to the contrary of [93]). This could be due to stress conditions, such as insufficient nutrient supply in the case of organic samples.

Peak 2 showed a molecular ion at m/z 625. Compound 2 was tentatively identified as apigenin-6/8-C-pentoside-8/6-C-hexoside in accordance with literature data.[94,95]

Peak 3. MS analysis showed a molecular ion at m/z 366 and a fragmentation pattern similar to those of di-C-glycosides. The MS/MS fragmentation gave CNLs of 180 (galactose or glucose), 162 (hexose), 120 (characteristic for a C-hexoside) and 42 (loss of CH_2CO group). On the basis of these results, a hexose-hexose-N-acetyl structure was proposed.

Peaks 4 with t_r = 27.8 and 29.3 contained the same molecular ion with $[M-H]^-$ = 563 characterized by the same complex of daughter ions indicating isomers. MS/MS data showed fragments at m/z 473 and 443, indicating the

presence of a C-hexosyl unit. Fragment at m/z 503 corresponding to the fragmentation of pentose. The ions at m/z 353 (aglycone+83) and 383 (aglycone+113) supported the conclusion that apigenin was the aglycone. Therefore, its general structure could be apigenin-6-C-pentoside-8-C-hexoside.[94-98]

Peak 5 corresponded to major molecular ion at m/z 468. The fragment ions included ions at m/z 332, 306, 161 and 289. We were not able to identify the compound. The similar compound (by molecular ion and fragmentation) was found in glabrous canary seed groats.[99]

Peak 6 showed a molecular ion at m/z 452. The MS/MS data showed fragments at m/z 306, 316, 135, 145 and 332. Fragment ion at m/z 306 refers to the deprotonated GSH moiety and CNLs of 120 and 146 indicating the presence of a rhamnoside group.

Peak 7 showed an intense molecular ion at m/z 285, respective compound was identified as luteolin.[97]

Peaks 2 and 7 are not represented in Figure 10. These were responsible for scattering in PCA results of flour and bran portions and the whole grains of three different varieties (Figure 9).

4. CONCLUSIONS

Comparative metabolomics of conventional vs. transgenic maize by CE-MS

- CE-TOF-MS can provide valuable information for assessing the existence (or not) of unexpected modifications. The results indicate that the unexpected variations that the metabolism of a transgenic organism seem to have compared to the wild line.
- A high number of metabolites have been identified in both isogenic and transgenic samples, and some of their main differences have been highlighted from the results of Student's t-test and PCA, involving differences in their quantity rather than in the nature or presence/absence of some of these compounds. For example, L-carnitine could be a possible biomarker of transgenic Bt maize.

Characterization and comparison of wheat varieties (*Triticum aestivum*) by LC-MS/MS

- It is demonstrated that oxylipins, products of free fatty acid peroxidation, can be identified in wheat grains by a relatively simple LC-MS/MS method using RP chromatographic columns and an aqueous formic acid/acetonitrile gradient as mobile phase.
- Several different oxylipins were identified, and it is suggested that, for example, 12,13-dihydroxy-9-octadecenoic acid (iso-LTX-diol), characterized in all of the grain samples by the highest oxylipin peak, could be a good candidate for a marker of lipid oxidation in the grains. Further investigations, including analysis after time intervals within the same harvest, are necessary to confirm this statement.
- Differences in the metabolomics patterns of wheat varieties could be individualized in results of PCA
- It can be assumed from PCA results that the identified oxylipins in the different wheat varieties are variety specific, involving differences in their quantities rather than in the nature or presence/absence of some of these compounds.
- It was found from PCA results that both the winter wheat grown in conventional conditions and the spring wheat grown in organic conditions, differ from spring wheat grown in conventional conditions of the high level of sugar content. It could be explained with an exposure of osmotic stress resistance.
- PCA results showed that only the bran fractions of the investigated varieties were significantly distinct from each other; it may refer to the dependence on the variety.

Characterization and comparison of wheat varieties (*Triticum aestivum*) by CE-DAD

- CE is a suitable and accurate method for the separation and quantification of phenolic compounds in wheat samples.
- The content of bound phenolic acids of winter wheat was found to be

significantly higher than that of spring wheat.

- The contents of total free phenolics of all winter wheat varieties were quite similar.
- The phenolic content of the wheat varieties grown in organic conditions was higher than that of the wheat varieties grown in conventional conditions.
- A higher content of phenolics possessed a higher antioxidant activity.

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APPENDIX I

Table 1. Metabolites found by CE-TOF-MS in the PR33P66 and PR33P66 Bt maize samples. (Publication I, pg 6333).

Table 1. Metabolites Found by CE-TOF-MS in the PR33P66 and PR33P66 Bt Maize Samples[a]

compound no.	$M_{r\ wild}$	$M_{r\ GMO}$	theoretical M_r	tentative compound	A_{GMO}/A_{wild}	refs
1	319.225	319.227	319.225	$C_{18}H_{29}N_3O_2$	3.393	
2	146.047	146.046	146.105	3,5-diaminocaproate	2.937	
3	264.103	264.103	264.101	subaphyllin	3.814	35
4	103.106	103.104	103.101	choline	2.354	17, 29, 30
5	174.116	174.115	174.113	arginine	1.273	31
6	155.075	155.072	155.071	histidine	1.782	17, 29, 31
7	135.060	135.057	135.054	adenine	3.309	
8	103.068	103.065	103.065	GABA	0.608	30
9	161.110	161.107	161.106	L-carnitine	14.427	
10	89.051	89.048	89.049	alanine	0.724	17, 30, 31
11	105.047	105.044	105.042	serine	0.726	31
12	117.085	117.083	117.080	valine	2.692	29−31
13	129.085	129.081	129.080	homoproline	1.702	
14	131.101	131.100	131.094	leucine	1.488	29, 31
15	137.054	137.055	137.049	trigonelline	1.964	17, 29−31
16	245.237	245.237	245.148	β-alanyl-L-arginine	2.245	
17	119.064	119.059	119.059	threonine	1.287	29, 31
18	115.073	115.070	115.065	proline	2.002	29, 31
19	147.059	147.056	147.054	glutamic acid	0.745	29−31
20	187.067	187.067	187.120	7-keto-8-aminopelargonic acid	1.239	32, 33
21	143.101	143.098	143.094	stachydrine	3.259	34
22	181.077	181.074	181.073	tyrosine	0.966	29, 31
23	133.043	133.041	133.039	aspartic acid	1.167	30, 31
24	437.228	437.234	437.231	lunarine	1.691	36
25	342.100	342.093	342.095	1-Caffeoyl-β-D-glucose	0.364	
26	279.084	279.085	279.089	graveoline	1.693	29
27	214.013	214.010	214.095	pyrimidine nucleoside	6.669	

[a] See text for details.

APPENDIX II

Table 2. Retention times (t_r), deprotonated molecular ions and fragment ions obtained from LC-MS analysis of metabolites in variety: Anthus. In the column ´Fragment ions´, base peaks are shown in bold.

Peak no	Tentative compound	$[M-H]^-$ (m/z)	t_r (min)	Fragment ions (m/z)
1	Dihexoside (unspecified)	341	1.5	**179**/161/143/119/131/101
2	Apigenin-6/8-C-pentoside-8/6-C-hexoside	625	11.9	**485**/179/221/383/323/341
3	Hexose-hexose-N-acetyl	366	23.3	**186**/204/142/246
4	Apigenin-6-C-pentoside-8-C-hexoside	563	27.8 29.3	**353**/383/443/473/503
5	Unknown	468	31.3	**332**/306/161/289
6	Rhamnoside	452	33.6	**306**/316/135/145/332
7	Luteolin	285	41.7	**241**/285/175/199/151

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ABSTRACT

This thesis describes the comparative metabolomics of different maize and wheat varietes using CE and HPLC for separation of metabolites with UV and MS detection and PCA for understanding the comprehensive data set and differentiation of the profiles of varieties.

Chapter 1 describes the essence of metabolomics, gives the overview and basics of current metabolite profiling techniques with illustrations of previous work by other research groups, and introduces the PCA application.

Chapter 2 contains the experimental part with information pertaining to the work.

Chapters 3 and 4 describe the results and conclusions of the work.

It was demostrated that in comparison to conventional and transgenic maize varieties grown under identical conditions, CE-TOF-MS can provide valuable information for assessing the existence (or not) of unexpected modifications. The results confirm that the metabolism of a transgenic organism seems to have unexpected variations compared to the isogenic wild line. A high number of metabolites were identified in both isogenic and transgenic samples, and some of their main differences were highlighted from the results of Student t-test and PCA. For example, L-carnitine and stachydrine were identified as overexpressed metabolites in all the studied genetically modified maize varieties; therefore these could be a possible biomarkers of transgenic Bt maize.

A total number of fifteen of wheat varieties were characterized and compared from different sides using CE and HPLC separation methods with UV and MS detection.

It is demonstrated that oxylipins can be identified in wheat grains by a relatively simple HPLC-MS method. Based on PCA results, the identified oxylipins in the different wheat varieties are variety specific. The several different oxylipins were identified, and it is suggested that, for example, iso-LTX-diol, characterized in all of the grain samples by the highest oxylipin peak, could be a good candidate for a marker of lipid oxidation in the grains.

The phenolic compounds and antioxidant activity of wheat samples were investigated by spectrofotometrically and with CE-UV method. The content of bound phenolic acids of winter wheat was found to be significantly higher than that of spring wheat. The contents of total free phenolics of all winter wheat varieties were found to be quite similar. The phenolic content of the wheat varieties grown in organic conditions was higher than that of the wheat varieties grown in conventional conditions. And the fact that the wheat extracts with a higher content of phenolics have a higher antioxidant activity was confirmed.

The metabolomic patterns of whole grains as well as the flour and the bran portions of the grain of different wheat varieties differentiated in the results of PCA. It was found that both the winter wheat grown in conventional conditions and the spring wheat grown in organic condition differ from spring wheat grown in conventional conditions of the high level of sugar content.

Also, PCA results showed that only the bran fractions of investigated varieties were significantly individuated from each other, it may refer to the dependence on the variety.

KOKKUVÕTE

Antud töö käsitleb teraviljasortide (mais ja nisu) võrdlevat metaboloomikat kasutades kapillaarelektroforeesi ja kõrgsurve vedelikkromatograafiat teraviljades sisalduvate metaboliitide lahutamiseks, ultraviolett (UV) ja mass-spektromeetrilist (MS) detekteerimist ning peakomponentide analüüsi (PCA) teraviljasortide metaboolsete profiilide uurimiseks.

Peatükk 1 kirjeldab metaboloomika olemust, annab ülevaate ning põhitõed metaboolsete profiilide uurimisel kasutatavatest meetoditest koos vastavate viidetega kirjandusele ning tutvustab PCA-d.

Peatükis 2 on toodud eksperimentaalne osa.

Peatükkides 3 ja 4 esitatakse tulemused ja järeldused. CE-TOF-MS rakendamine võib osutuda väga väärtuslikuks ootamatute modifikatsioonide ilmnemise (või siis puudumise) hindamisel tava- ja transgeense maisi sortide võrdlemisel. Tulemused kinnitavad, et transgeensete organismide metabolism erineb mõnevõrra neile vastavatest tavaliinidest. Töö käigus identifitseeriti 27 metaboliiti isogeensetes ja transgeensetes proovides, ning peamised erinevused ilmnesid Studenti t-testi ja PCA rakendamisel. Näiteks, L-karnitiin ja stahhüdriin identifitseeriti kui üle-ekspresseeritud metaboliidid kõikide transgeensete maisi proovide korral, seetõttu võiks neid käsitleda kui võimalikke biomarkereid transgeense Bt maisi puhul.

Töös uuriti ja võrreldi kokku 15 erinevat nisusorti kasutades CE ja HPLC koos UV ja MS detekteerimisega.

Leiti, et oksülipiine on võimalik identifitseerida suhteliselt lihtsa HPLC-MS meetodiga. PCA tulemuste põhjal võib öelda, et identifitseeritud oksülipiinide sisaldus erinevates nisu sortides on sordipõhine. Esile on tõstetud iso-LTX-diool'i esinemine (mis oli intensiivseim oksülipiini piik kõikide proovide korral), mida võiks pidada lipiidide oksüdatsiooni markeriks nisu puhul.

Nisuproovide fenoolsete ühendite sisaldust ja antioksüdatiivsust hinnati spektrofotomeetriliselt ja kasutades CE-UV meetodit. Leiti, et seotud fenoolsete ühendite sisaldus on talinisu korral märkimisväärselt kõrgem kui suvinisu proovides. Märkimisväärset erinevust vabalt esinevate fenoolsete ühendite sisalduse puhul talinisude korral ei täheldatud. Mahe nisu proovide puhul leiti fenoolsete ühendite sisaldus kõrgeim olevat.

Samuti leiti, et kõrgema polüfenoolide sisaldusega proovidel on ka kõrgem antioksüdatiivsus.

PCA tulemustest lähtuvalt võib öelda, et nisu erinevate sortide ja nisuterade erinevate fraktsioonide (täistera, jahu ja klii) metaboloomsed profiilid on erinevad. Täheldati, et peamiselt talinisu ja mahe suvinisu erinevad suvinisust (tavatingimustes kasvatatud) kõrgema suhkrusisalduse poolest.

Samuti võib PCA tulemustest lähtuvalt öelda, et erinevate sortide klii fraktsioonid on märkimisväärselt erinevad, mis viitab sordipõhisusele (mida ei saa aga väita nt. eri sortide jahu fraktsioonide kohta).

ORIGINAL PUBLICATIONS

ARTICLE I

T. Levandi, C. Leon, M. Kaljurand, V. Garcia-Canas, A. Cifuentes. Capillary electrophoresis time-of-flight mass spectrometry for comparative metabolomics of transgenic versus conventional maize. Anal. Chem. 2008, 80, 6329-6335.

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Capillary Electrophoresis Time-of-Flight Mass Spectrometry for Comparative Metabolomics of Transgenic versus Conventional Maize

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In this work, capillary electrophoresis time-of-flight mass spectrometry (CE-TOF-MS) is proposed to identify and quantify the main metabolites in three lines of genetically modified (GM) maize and their corresponding nontransgenic parental lines grown under identical conditions. The shotgun-like approach for metabolomics developed in this work includes optimization of metabolite extraction from GM and non-GM maize, separation by CE, online electrospray-TOF-MS analysis, and data evaluation. A large number of extraction procedures and background electrolytes are tested in order to obtain a highly reproducible and informative metabolomic profile. Thus, using this approach, significant differences were systematically observed between the detected amounts of some metabolites in conventional varieties (Aristis, Tietar, and PR33P66 maize) compared with their corresponding transgenic lines (Aristis Bt, Tietar Bt, and PR33P66 Bt maize). Results point to some of these metabolites as possible biomarkers of transgenic Bt maize, although a larger number of samples needs to be analyzed in order to validate this point. It is concluded that metabolomics procedures based on CE-TOF-MS can open new perspectives in the study of transgenic organisms in order to corroborate (or not) their substantial equivalence with their conventional counterparts.

Nowadays, the use of genetically modified organisms (GMOs) has seen a great increase in agriculture and food industry. Thus, genetic engineering can be used, for example, to improve resistance of crops to plagues or pesticides, to provide better nutritional properties, etc.[1,2]

In spite of the mentioned advantages, the use of GMOs in foods is not commonly accepted in many countries.[3] This situation has led to the implementation in some countries of different regulations regarding the development, growing, or commercialization of genetically modified products.[4,5] As a result, nowadays, research on how the different genetic modifications can impact on the chemical composition is of great interest since the existence of unexpected modifications in GMOs has already been demonstrated.[6] In this regard, a mixed strategy is usually applied by the biotech companies and regulatory laboratories to assess the safety equivalence between transgenic and parental nontransgenic organisms (maize or soy, for instance) including field investigations, animal nutrition, and basic chemical composition studies. However, it has repeatedly been demonstrated that these strategies are not very useful to detect unexpected modifications in GMOs.[6] Moreover, the mentioned strategies devised to study the nutritional, safety assessment, and chemical composition of the first GMO generation will be much more difficult to apply to the coming new generation of GMOs in which significant changes in other constituents have been deliberately introduced (for example, increasing fatty acids, amino acid content, polyphenols, vitamins, or reducing undesirable constituents), requiring the development of more powerful and informative analytical procedures.[7–11]

In this regard, the use of DNA-based techniques has shown impressive possibilities to sensitively detect GMOs in foods.[12–16]

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However, these techniques mainly provide results on the existence (or not) of GMOs without offering any additional information on the composition of the investigated GMOs. Other analytical techniques such as NMR,[17,18] HPLC,[19] or CE[20] have also shown interesting possibilities to classify transgenic maize[17–19] or soybean[19] based on their metabolites or protein profiles. Moreover, the combined use of GC/MS and LC/MS has also shown interesting possibilities to investigate the substantial equivalence between genetically modified and conventional potatos.[21] These techniques have been applied either to compare general profiles from transgenic versus nontransgenic foods (i.e., no identification of the analytes was carried out[17,19,20] or to establish a more sound comparison including the identification of some representative analytes.[18,21] However, to our knowledge, the possibilities of CE−MS for metabolomics of GMOs have not been investigated so far.

In this work, an original analytical strategy is proposed able to provide information on the composition of transgenic maize based on metabolomics studies by CE−MS of several transgenic varieties followed by a comparison with their corresponding isogenic wild lines. The goal of this work is, therefore, to carry out a comparative profiling of metabolites found in transgenic maize varieties versus their corresponding isogenic wild lines grown under identical conditions. To do this, a complete analytical strategy is developed that combines metabolites extraction from maize samples, separation by capillary electrophoresis and chemical characterization by online electrospray time-of-flight mass spectrometry (CE-TOF-MS). Resulting CE−MS electropherograms are statistically evaluated by pair-wise comparison of peak areas by simple Student's *t*-test and principal component analysis (PCA) of a whole data set. L-Carnitine and stachydrine are identified as overexpressed metabolites in all the studied genetically modified maize varieties.

MATERIALS AND METHODS

Chemicals. All chemicals were of analytical reagent grade and used as received. Methanol and acetonitrile from Scharlau (Barcelona, Spain), chloroform from SDS (Peypin, France), acetone from Labscan (Dublin, Ireland), and ethanol from Prolabo (Fountenay sous Bois, France) were used as extraction buffers. Ammonium hydrogen carbonate from Fluka (Buchs, Switzerland), formic acid from Riedel-de Haën (Seelze, Germany), acetic acid from Merck (Darmstadt, Germany), ammonium hydrogen acetate from Panreac Química S.A. (Barcelona, Spain), and 2-propanol from Scharlau, were used for the CE running buffers and sheath liquids. The buffers were stored at 4 °C and warmed at room temperature before use. A water solution containing 0.1 mol/L sodium hydroxide from Panreac Química S.A. and 1% SDS from Fluka was used for capillary washing before each analysis. Distilled water was deionized by using a Milli-Q system from Millipore (Bedford, MA).

Maize Samples. Obtainment and Characterization. The investigated varieties of conventional and transgenic maize were obtained from a field assay carried out in Estación Experimental Agrícola Mas Badía in Tallada d'Empordá (Girona, Spain) using commercial varieties. Namely, in order to skip any influence from the growing conditions, Aristis maize (wild type and its Bt transgenic variety), Tietar maize (wild type and its Bt transgenic variety), and PR33P66 maize (wild type and its Bt transgenic variety) were grown under the same field conditions and investigated in this work. The transgenic and no transgenic nature of all these maize samples was confirmed based on their DNA using an analytical procedure developed in our laboratory and described elsewhere.[12–16]

Extraction Procedure. The extraction procedure was optimized in order to obtain a fraction from maize flour highly informative in a reproducible way. In order to do that, CE-UV (see below) was used to monitor the extraction process. Different solvents (water, methanol, ethanol, acetonitrile, acetone), solvent mixtures, and sample amounts were tried in order to optimize the extraction. Namely, 0.1, 0.5, or 2 g of maize flour were weighed and extracted using 3, 6, or 10 mL of solvent (methanol, acetonitrile, acetone, water, and mixtures of ethanol/water, methanol/water with two different ratios, 50:50 and 75:25, in an ultrasonic bath for 10 min. After sonication, samples were centrifuged for 5 min at 3000 rpm and liquid phases were filtered through a 0.45-μm filter. Liquid phases were taken to dryness in a rotatory evaporator and redissolved in 0.5 mL of solvent before injection. For comparison,[17,22] a different extraction procedure was also tried, adding 3 mL of methanol/chloroform mixture (2:1) to the flour of maize. The samples were stirred, and 1 mL of chloroform and 1.2 mL of water were added. Samples were stored at 4 °C for 1 h and then centrifuged at 10000*g* for 20 min at 4 °C. The resulting upper hydroalcoholic and lower chloroformic phases were separated. The extraction procedure was performed twice and thereafter dried in rotary evaporator and redissolved in 1 mL of methanol.

The selected extraction conditions were as follows: About 2.0 g of maize flour was weighed out and extracted with 10 mL of methanol/water mixture (50:50) in an ultrasonic bath for 10 min. After sonication, samples were centrifuged for 5 min (3000 rpm) and liquid phases were filtered through a 0.45-μm filter. Liquid phases were taken to dryness in a rotary evaporator, redissolved in 0.5 mL of methanol/water (50:50), and injected into the CE system.

CE-UV Conditions. Analyses were carried out with a P/ACE 2100 (Beckman Instruments, Fullerton, CA) CE equipment with UV detection. CE instrument was controlled by a PC running the system GOLD software from Beckman. An uncoated fused-silica capillary with dimensions 47-cm total length, 40-cm effective length, 50-μm i.d., and 375-μm o.d. was used. Initially, a new capillary was preconditioned by rinsing with 1 mol/L NaOH for 20 min, followed by a 10-min rinse with Milli-Q water. At the

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beginning and at the end of each day, the capillary was washed with 1.0 mol/L NaOH and water for 5 min and electrolyte solution for 15 min. Between runs, the capillary was reconditioned with the electrolyte solution for 3 min. Sixteen different background electrolytes (BGE) compatible with CE−MS and with pH values ranging from 1.9 to pH 8.24 were first tested by CE-UV in order to optimize the separation of metabolites. The 16 BGEs were composed (depending on the pH) of different combinations of formic acid, acetic acid, methanol, ethanol, acetonitrile, ammonium hydrogencarbonate, and ammonium hydroxide. The applied voltage was 20 kV; samples were injected hydrodynamically (22 nL, 15 s at 0.5 psi) and detected at 200 and 280 nm.

CE-ESI-TOF-MS Conditions. Analyses were carried out with a P/ACE 5010 CE apparatus from Beckman Instruments coupled with an orthogonal electrospray interface (ESI, model G1607A from Agilent Technologies, Palo Alto, CA) to a time-of-flight microTOF MS detector from Bruker Daltonik (Bremen, Germany). An uncoated fused-silica capillary with 50-μm i.d., 375-μm o.d., and 80 cm of total (and detection) length was used. CE washing protocol, CE conditions, and sample injection were the same as the optimized by CE-UV. The nebulizing gas and voltage were stopped during the washing procedure. Electrical contact at the electrospray needle tip was established via a sheath liquid delivered at a flow rate of 0.24 mL/h by a 74900-00-05 Cole Parmer syringe pump (Vernon Hills, IL). The mass spectrometer was operated in the 50−450 m/z range during separation. Nebulizing gas, dry gas flow rate and dry gas temperature were varied, as well as the nature of the sheath liquid and its flow rate in order to increase the sensitivity of the detection (vide infra).

Data Analysis. Compounds were tentatively identified using the DataAnalysis 3.4 (from Bruker Daltonics) to generate the molecular formula, introducing this formula in different databases such as Kegg, Chemspider, or PubChem. Assignments were further corroborated using the isotopic pattern simulation from DataAnalysis 3.4. Electrophoretic mobility of the assigned compounds was used to further corroborate the identification.

To detect statistically significant peak area differences, a Student's t value was initially calculated as follows:[23]

$$t_{exp} = \frac{|A_i^W - A_i^{GMO}|}{\sqrt{(s_i^W)^2 + (s_i^{GMO})^2}}\sqrt{n} \quad (1)$$

Here A_i^W is ith peak area for wild variety and $s_i^W$is its standard deviation. Index GMO denotes peak area and standard deviation for genetically modified variety and n is number of measurements. Since $n = 3$ in this work, the number of degrees of freedom is $2 \times 3 - 2 = 4$ and critical two-tailed Student's t value for 99% confidence level is $t_{crt} = 4.6$.

PCA was carried out using Matlab (Mathworks, Natick MA) environment by standard singular value decomposition procedure. For data preprocessing, peak areas were replaced by their logarithms (to reduce influence of large peaks) and mean centered. In PCA, a data matrix, d, is decomposed to the scores matrix t and loading matrix p as follows: $d = tp'$, where $'$ denotes transpose. In this decomposition d matrix contains CE−MS data points in new, principal component axes, and t matrix contains principal components coordinates.

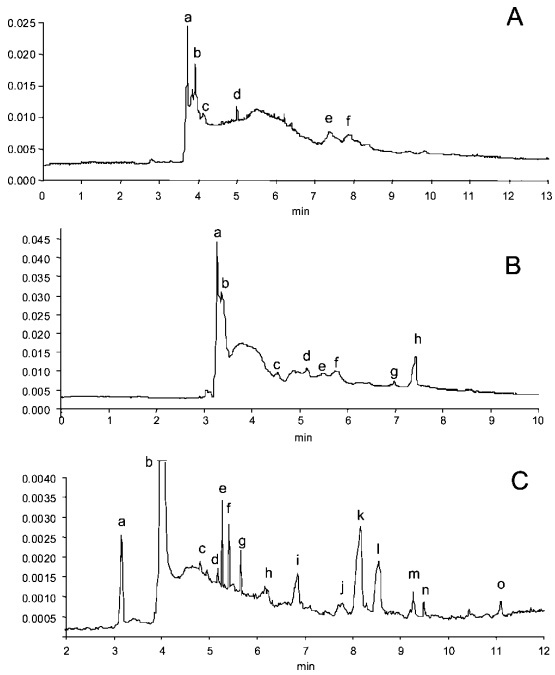

Figure 1. CE-UV electropherograms of metabolites from Aristis maize obtained using for the extraction: (A) water, (B) EtOH/H_2O (50:50), and (C) MeOH/H_2O (50:50). Other conditions, run voltage 20 kV, injection time 5 s, capillary 50 cm (43 cm, effective length) and 50 μm i.d.; BGE, 25 mM NH_4HCO_3 buffer at pH 8.08; detection wavelength, 200 nm. Letters a, b, c, etc., correspond to reproducible peaks observed in two different extracts obtained and analyzed under identical conditions.

RESULTS AND DISCUSSION

Samples from three genetically modified lines of maize and their respective wild (conventional) varieties of maize grown under the same conditions were studied in this work. Genetic modification on transgenic maize was based on the inclusion of the Cry1Ab gene, which provides resistance to some worm plagues through the synthesis of a Bt protein.[24]

Optimization of the Metabolite Extraction from Maize Samples. It is assumed that extraction is a fundamental point in any analytical method. This step becomes even more critical in the case of metabolomic approaches since the extraction conditions will induce an initial trend on the type of metabolites finally identified based on the polarity of the solvent (or mixture of solvents) used to carry out the extraction. The possibility to obtain a good extraction procedure of metabolites from maize flour was deeply explored using CE-UV to monitor at 200 and 280 nm the results of the extraction. To do this, the different extraction conditions described above were investigated in order to determine the extraction method that led to a higher number of peaks

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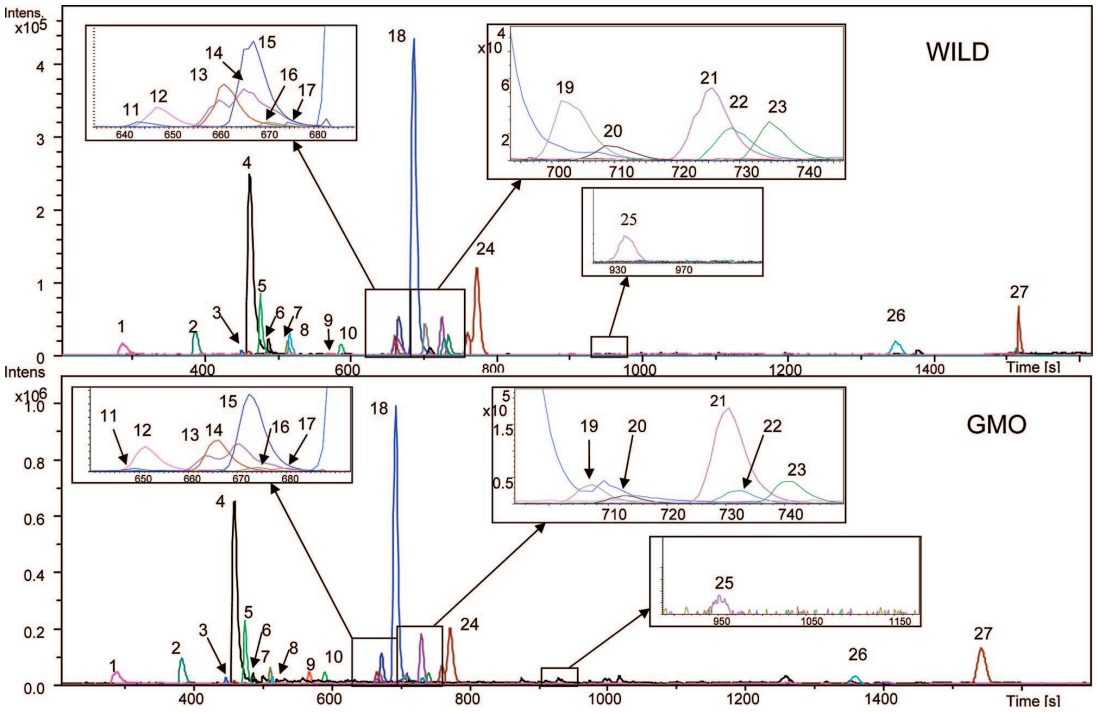

Figure 2. CE-TOF-MS extracted ion electropherograms of the 27 metabolites detected in PR33P66 and PR33P66 Bt maize. Experimental conditions: BGE composed of 5% formic acid at pH 1.90, total length of the capillary 80.0 cm; 50 μm i.d.; applied voltage 20 kV; volume injected 22 nL; temperature 30 °C. Sheath liquid 2-propanol/water (50:50, v:v), at 0.24 mL/h, nebulizer pressure was 0.4 bar and dry gas conditions 4 L/min N_2 at 180 °C. MS scan range from 50 to 450 *m/z*.

in a reproducible way, assuming this means a higher number of metabolites extracted. Two different maize samples were selected for this initial extraction study, a transgenic maize (Aristis Bt), and its corresponding isogenic line, grown in the same field conditions (Aristis). As the aim of this part was to extract the highest number of metabolites, extraction solutions able to cover a wide range of solvents (and polarities) were frequently used, including ethanol/water, methanol/chloroform, and methanol/water mixtures. In order to optimize the extraction, the [amount of sample/volume of solvent] ratio was also considered, varying the amount of sample from 0.5 to 2 g and the solvent extraction volume from 3 to 10 mL. The best results in terms of number of peaks were obtained using 2 g of sample in 10 mL of methanol/water (50:50, v:v) with ultrasounds for 10 min. Figure 1 shows some representative examples of the CE-UV electropherograms obtained during the optimization of the metabolites extraction. Namely, Figure 1 shows the CE-UV electropherogram obtained using water (Figure 1A), EtOH/water (50:50, v:v; Figure 1B) and MeOH/water (50:50, v:v; Figure 1C). The best results were obtained using MeOH/water during the extraction as corroborated by Figure 1C. Considering these optimum conditions, the next step in this analytical development was to optimize the CE separation using the selected maize extracts here obtained.

CE-UV Separation. The objective in this part of the work was to obtain a BGE compatible with the subsequent ESI-MS analysis and able to provide a fast separation of metabolites with high resolution and sensitivity. Thus, 16 BGEs at different pHs were tested at this stage in order to achieve adequate CE separation conditions. The pH of the buffers varied from 1.9 to 8.24, and other experimental CE conditions (namely, separation voltage, temperature, and injection time) were also evaluated in order to improve the separation.

The results obtained from this systematic study allowed us to select the following conditions: two BGEs were selected, 2.5% formic acid buffer at pH 1.9, and a second composed of 25 mM NH_4HCO_3 buffer in 10% methanol at pH 7.82. The injection volume and the voltage were optimum at 22 nL s and 20 kV, respectively, and the temperature was set at 30 °C. Under these conditions, good separations were obtained for both buffers with a relatively high number of peaks and short times (less than 10 min, in both cases). This result is especially interesting considering the longer capillaries usually required for coupling CE and MS instruments.

CE-ESI-TOF-MS Analysis. Once the CE conditions were optimized, the next step was to optimize the ESI-TOF-MS parameters in order to obtain good sensitivity keeping the resolution as high as possible, improving in this way the identification capability of our system. For this optimization, the same extracted samples used in the CE-UV experiments were used. The two BGE selected above were tested together with different sheath liquids. It was systematically observed that the number of compounds and sensitivity provided by the acidic buffer were in all cases better, and therefore, these conditions were selected for the subsequent optimization. Namely, a 5% formic acid, at the same pH other than the optimum (pH = 1.9) was finally used since it was observed to favor slightly the resolution without increasing in excess the analysis time.

Table 1. Metabolites Found by CE-TOF-MS in the PR33P66 and PR33P66 Bt Maize Samples[a]

compound no.	$M_{r\ wild}$	$M_{r\ GMO}$	theoretical M_r	tentative compound	A_{GMO}/A_{wild}	refs
1	319.225	319.227	319.225	$C_{18}H_{29}N_3O_2$	3.393	
2	146.047	146.046	146.105	3,5-diaminocaproate	2.937	
3	264.103	264.103	264.101	subaphyllin	3.814	35
4	103.106	103.104	103.101	choline	2.354	17, 29, 30
5	174.116	174.115	174.113	arginine	1.273	31
6	155.075	155.072	155.071	histidine	1.782	17, 29, 31
7	135.060	135.057	135.054	adenine	3.309	
8	103.068	103.065	103.065	GABA	0.608	30
9	161.110	161.107	161.106	L-carnitine	14.427	
10	89.051	89.048	89.049	alanine	0.724	17, 30, 31
11	105.047	105.044	105.042	serine	0.726	31
12	117.085	117.083	117.080	valine	2.692	29−31
13	129.085	129.081	129.080	homoproline	1.702	
14	131.101	131.100	131.094	leucine	1.488	29, 31
15	137.054	137.055	137.049	trigonelline	1.964	17, 29−31
16	245.237	245.237	245.148	β-alanyl-L-arginine	2.245	
17	119.064	119.059	119.059	threonine	1.287	29, 31
18	115.073	115.070	115.065	proline	2.002	29, 31
19	147.059	147.056	147.054	glutamic acid	0.745	29−31
20	187.067	187.067	187.120	7-keto-8-aminopelargonic acid	1.239	32, 33
21	143.101	143.098	143.094	stachydrine	3.259	34
22	181.077	181.074	181.073	tyrosine	0.966	29, 31
23	133.043	133.041	133.039	aspartic acid	1.167	30, 31
24	437.228	437.234	437.231	lunarine	1.691	36
25	342.100	342.093	342.095	1-Caffeoyl-β-D-glucose	0.364	
26	279.084	279.085	279.089	graveoline	1.693	29
27	214.013	214.010	214.095	pyrimidine nucleoside	6.669	

[a] See text for details.

The optimum conditions were 2-propanol/water (50:50, v/v) as sheath liquid flowing at 0.24 mL/h together with 0.4 bar N_2 as nebulizing gas and dry gas at 180 °C and 4 L/min. Other TOF settings were as follows: 50 μs for the transfer time and 5 μs as prepulse storage. The MS scan range was fixed in a range that allowed detecting as many metabolites as possible without reducing sensitivity; namely, the scan range from 50 to 450 m/z was selected.

The reproducibility of the method was evaluated by measuring the migration time and area of three selected peaks injecting in triplicate three samples obtained under the same extraction conditions. The $RSD_{n=9}$ values were lower than 1.5% for the migration time and lower than 12% for the peak areas.

Metabolite Search and Identification. Once the extraction and CE-TOF-MS conditions were optimized, the six maize samples were analyzed, injecting all of them in triplicate. An example of the CE-TOF-MS extracted ion electropherogram obtained for two of the samples (PR33P66, PR33P66 Bt) is shown in Figure 2. As can be seen, 27 metabolites were easily found and tentatively identified as indicated in Table 1. Tentative identification was carried out based on the highly accurate mass determination provided by CE-TOF-MS, which allowed generating a more probable molecular formula for each metabolite. This molecular formula was then introduced in, for example, Kegg, Chemspider, or PubChem databases to obtain the metabolite identification. These assignments were corroborated using isotopic pattern simulations. Moreover, the expected electrophoretic mobility of each compound at the separation pH was also used to further corroborate the assignments. Thus, at the separation pH of 1.9 used for the CE-TOF-MS electropherograms of Figure 2, all the amino groups will be positively charged, while the acid groups will be practically neutral or scarcely ionized, giving a global positive charge to all the metabolites that share these two groups in their molecule (for example, amino acids), allowing their migration toward the MS detector under the separation voltage applied.

Thus, as expected, compounds with a higher number of amino groups in their structure such as 3,5-diaminocaproate, subaphyllin, or arginine are observed to migrate first. A second group of metabolites migrates subsequently formed by different amino acids (GABA, carnitine, alanine, serine, valine, leucine, threonine, proline), including other metabolites such as trigonelline, a product from the metabolism of vitamin B_3,[21] or amino acid derivatives such as homoproline or small peptides like β-alanyl-L-arginine, with lower charge/mass ratio and, therefore, lower mobility. The next migrating group is formed by less positive amino acids such as tyrosine, glutamic acid, and aspartic acid as well as other acidic metabolites such as 7-keto-8-pelargonic acid and stachydrin, with a higher ratio of oxygen in their molecules. This leads to higher migration times, due to the charge compensation induced by the carboxylic groups. Finally, a group of alkaloids (as lunarine or graveoline) together with a nucleoside and a polyphenol, all with higher oxygen content in their structure, are detected at the end of the separation. This behavior is explained considering that these compounds, although still bearing some positive electrical charge, show a very low positive character due to the higher amount of negatively ionizable groups in the molecule that partially compensate their positive charge.

Tables 2 and 3 show the results for the other two couples of maize, Aristis/Aristis Bt and Tietar/Tietar Bt, respectively. The 27 compounds found in the 6 samples are listed in Tables 1−3. In these tables, a comparison between the relative molecular mass values found for metabolites in the transgenic ($M_{r\ GMO}$) and the wild maize ($M_{r\ wild}$) is provided, together with a comparison with

Table 2. Metabolites Found by CE-TOF-MS in the Aristis and Aristis Bt Maize Samples

compound no.	$M_{r\ Wild}$	$M_{r\ GMO}$	theoretical M_r	tentative compound	A_{GMO}/A_{wild}	refs
1	319.217	319.221	319.225	$C_{18}H_{29}N_3O_2$	0.790	
2	146.035	146.041	146.105	3,5-diaminocaproate	2.030	
3	264.091	264.097	264.101	subaphyllin	1.019	35
4	103.098	103.104	103.101	choline	1.010	17, 29, 30
5	174.104	174.111	174.113	arginine	0.734	31
6	155.062	155.069	155.071	histidine	1.013	17, 29, 31
7	135.049	135.057	135.054	adenine	0.699	
8	103.057	103.064	103.065	GABA	0.653	30
9	161.100	161.105	161.106	L-carnitine	10.380	
10	89.042	89.046	89.049	alanine	1.093	17, 30, 31
11	105.039	105.041	105.042	serine	1.075	31
12	117.078	117.078	117.080	valine	1.550	29−31
13	129.079	129.078	129.080	homoproline	0.542	
14	131.095	131.094	131.094	leucine	1.002	29, 31
15	137.051	137.051	137.049	trigonelline	1.251	17, 29−31
16	245.232	245.228	245.148	β-alanyl-L-arginine	1.259	
17	119.058	119.058	119.059	threonine	0.724	29, 31
18	115.072	115.071	115.065	proline	0.990	29, 31
19	147.054	147.051	147.054	glutamic acid	0.617	29−31
20	187.061	187.059	187.120	7-keto-8-aminopelargonic acid	0.932	32, 33
21	143.096	143.096	143.094	stachydrine	8.787	34
22	181.072	181.070	181.073	tyrosine	0.848	29, 31
23	133.039	133.037	133.039	aspartic acid	0.978	30, 31
24	437.234	437.229	437.231	lunarine	1.862	36
25	342.105	342.097	342.095	1-Caffeoyl-β-D-glucose	2.058	
26	279.085	279.080	279.089	graveoline	0.505	29
27	214.011	214.010	214.095	pyrimidine nucleoside	1.835	

Table 3. Metabolites Found by CE-TOF-MS in Tietar and Tietar Bt Maize Samples

compound no.	$M_{r\ Wild}$	$M_{r\ GMO}$	theoretical M_r	tentative compound	A_{GMO}/A_{wild}	refs
1	319.230	319.219	319.225	$C_{18}H_{29}N_3O_2$	1.071	
2	146.043	146.041	146.105	3,5-diaminocaproate	1.611	
3	264.104	264.095	264.101	subaphyllin	1.047	35
4	103.102	103.098	103.101	choline	0.772	17, 29, 30
5	174.113	174.107	174.113	arginine	1.589	31
6	155.071	155.066	155.071	histidine	0.972	17, 29, 31
7	135.056	135.051	135.054	adenine	0.786	
8	103.063	103.060	103.065	GABA	0.456	30
9	161.107	161.100	161.106	L-carnitine	4.631	
10	89.046	89.043	89.049	alanine	0.652	17, 30, 31
11	105.040	105.039	105.042	serine	0.872	31
12	117.081	117.079	117.080	valine	0.797	29−31
13	129.081	129.076	129.080	homoproline	0.659	
14	131.096	131.090	131.094	leucine	0.993	29, 31
15	137.083	137.048	137.049	trigonelline	0.911	17, 29−31
16	245.237	245.226	245.148	β-alanyl-L-arginine	0.563	
17	119.060	119.055	119.059	threonine	0.908	29, 31
18	115.072	115.064	115.065	proline	0.683	29, 31
19	147.054	147.050	147.054	glutamic acid	1.116	29−31
20	187.064	187.057	187.120	7-keto-8-aminopelargonic acid	0.569	32, 33
21	143.097	143.093	143.094	stachydrine	2.509	34
22	181.074	181.065	181.073	tyrosine	0.807	29, 31
23	133.039	133.037	133.039	aspartic acid	1.236	30, 31
24	437.239	437.221	437.231	lunarine	1.225	36
25	342.105	342.094	342.095	1-caffeoyl-β-D-glucose	1.005	
26	279.085	279.076	279.089	graveoline	0.518	29
27	214.011	214.000	214.095	pyrimidine nucleoside	1.130	

the theoretically expected values, observing in all cases a good agreement. Moreover, the ratio between the peak area of each compound determined in the transgenic and wild maize is also included (A_{GMO}/A_{wild}). This ratio could indicate possible detected metabolites that are under- or overexpressed in genetically modified maize varieties. Using the Student's *t* test as described in Data Analysis (vide supra), two compounds (peaks **9** and **21**) were found to have statistically significant differences in peak areas in all varieties (i.e $t_{exp} > 4.6$). Namely, metabolite **9** with a molecular weight of 161.106, identified as L-carnitine, is clearly overexpressed in all the GMO samples. Thus, L-carnitine could be considered as a possible biomarker for the detection of transgenic samples. L-Carnitine is involved in the fatty acid metabolism, enhancing the transport of fatty acids inside the mitochondria for their oxidation, but also in the glucose metabolism favoring the glycogen storage.[26−28] Besides, compound **21**, with a molecular weight of 143.094 and identified as stachydrine, also called L-proline−betaine, could also be used as a potential

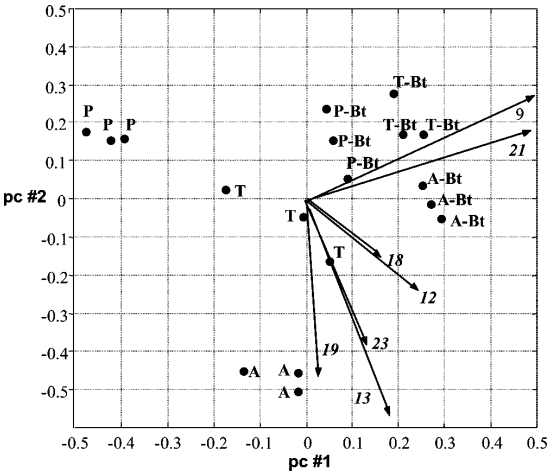

Figure 3. Representation of the CE−MS electropherograms in the first principal component coordinates (accounting of the 70% variability). "A" stands for conventional Aristis maize. "P" for conventional PR33P66 maize, and "T" for conventional Tietar maize, while the terms, "A-Bt", "P-Bt", and "T-Bt" correspond to their respective transgenic varieties. Vectors are loadings and numbers 9, 12, 13, 18, 19, 21 and 23 refer to peaks in CE−MS electropherograms.

biomarker, since it is overexpressed in all the three GMO samples. Although a larger number of samples needs to be analyzed in order to confirm this point, it is interesting to remark that the statistically significant difference was observed in all the investigated samples for only 2 compounds out of the 27 metabolites identified.

Thus, other two compounds differing more than statistically expected are metabolite **19** (identified as glutamic acid) in variety Aristis and **13** (identified as homoproline) in both varieties PR33P66 and Aristis. However, since the statistically significant difference for metabolites **13** and **19** is not systematically observed for all the samples, their difference can more likely be explained through natural variability.

Principal Component Analysis. To confirm the conclusions obtained from the elementary statistical analysis discussed above, the data as a whole were next subjected to PCA. The data matrix has dimensionality 18×7 (seven CE−MS peak areas chosen from electropherograms of six varieties, run in triplicate), data analysis was carried out as described in the Experimental Section. In Figure 3, CE−MS data are represented in two first principal component coordinates that account ~70% of data variability. Each point represents one particular electropherogram. As can be seen in Figure 3, conventional (A, P, and T points) and transgenic (A-Bt, P-Bt, and T-Bt points) varieties are nicely separated using this approach. To find out what feature (peak) is responsible for the separation of samples (i.e., sorting), loading vectors located in columns of p matrix are also represented in Figure 3. It is evident from Figure 3 that vectors corresponding to peaks **9** and **21** are the main ones responsible for the separation of wild and transgenic varieties. Therefore, this PCA confirms the straightforward statistical analysis performed above.

CONCLUSIONS

In this work, it is demonstrated that the development of metabolomics approaches based on capillary electrophoresis time-of-flight mass spectrometry to investigate transgenic foods, apart from having interest from a nutritional and food safety point of view, can provide additional information for assessing the existence (or not) of unexpected modifications in other metabolic pathways. Thus, this methodology can highlight details about the changes induced (if any) on other metabolic networks by the presence of significant modifications in unexpected compounds that could be linked after applying a "bottom-up" strategy to the genetic manipulation of the original genome.

All these results confirm that the metabolism of a transgenic organism seems to have unexpected variations compared to the wild line as already indicated by other authors.[6,29] In this work, a high number of metabolites have been identified in both isogenic and transgenic samples, and some of their main differences have been highlighted, involving differences in their quantity rather than in the nature or presence/absence of some of these compounds. It is suggested that, for example, L-carnitine could be a good candidate to be selected as biomarker of transgenic Bt maize, although a larger number of samples need to be analyzed in order to confirm this point.

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ARTICLE II

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Research Paper

Oxidation products of free polyunsaturated fatty acids in wheat varieties

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Oxygenated fatty acids (oxylipins) are secondary metabolites of polyunsaturated fatty acids (PUFA). Here, we present a novel high-performance liquid chromatograpic separation on a reversed-phase column (RP-HPLC) coupled with electrospray ionization-tandem mass spectrometry (ESI-MS/MS) for the determination of various (per)oxidation products of linoleic (*cis*,*cis*-9,12-octadecadienoic) acid in eight different varieties (four spring and four winter varieties) of wheat (*Triticum aestivum*). The procedure includes extraction of oxylipins, chromatograpic separation using a linear gradient of aqueous formic acid and acetonitrile, with subsequent identification of compounds by MS/MS. Among the identified oxylipins, leukotoxin (LTX)-diol and its isomer (iso-LTX-diol) are known as potentially toxic substances. The obtained data was used further for comparison of different wheat varieties by principal component analysis (PCA). From the results of PCA, differences can be observed in the patterns of wheat varieties.

Keywords: HPLC-MS/MS / Iso-LTX-diol / LTX-diol / Oxylipins / PCA / Wheat

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1 Introduction

Plant oxygenated fatty acids (oxylipins) are a diverse family of secondary metabolites that are produced by oxidative metabolism of polyunsaturated fatty acids (PUFA) *via* the addition of molecular O_2. An increased production of oxylipins is a characteristic response to pathogenesis, wounding and herbivores. Hence, it has become evident that various types of oxylipins participate in the regulation of many innate defense-related and developmental processes in plants. Some of these have direct antimicrobial or anti-insect functions [1], while others, especially the jasmonates and the recently discovered diverse cyclopentenone [2–6] lipids, are potent regulators of defense mechanisms, for example by stimulating proteinase inhibitors or by promoting the accumulation of antimicrobial secondary metabolites (phytoalexins). Research on oxylipins has focused mainly on the biosynthesis of the plant signaling molecule jasmonic acid and its role in the regulation of developmental and defense-related processes [2, 7–12]. Increasing evidence indicates that the collective biological importance of oxylipins in plants is also essential for the resistance of plants to pathogens, and it is comparable to that of the eicosanoid family of lipid mediators in animals.

A majority of the PUFA, released during lipolysis, belong to the groups of linoleic (18:2) and linolenic (18:3) acids. In the free form, these acids are especially sensitive to both free radical and enzymatic oxidation, mainly by lipoxygenases (LOX), although a group of cytochromes P450 specialized for the metabolism of hydroperoxy fatty acids and pathogen-induced oxygenases have also minor roles. The intermediate and end products of these multi-step oxidation processes are less unsaturated or saturated fatty acids, such as hydroxyoctadecaenoic acids (HODE) and other oxylipins. They may contain up to three hydroxyl groups in their molecules [13].

Current methods available for the study of hydroxy fatty acids involve mainly the use of gas chromatography (GC) coupled to mass spectrometry (MS), as underscored by libraries in use with abundant collections of GC-MS spectra of various derivatives and their relative retention times in GC [14–17]. Liquid chromatography (LC) is much less used [18, 19]. However, LC-MS/MS might be a powerful tool and complementary to GC-MS analysis as, *e.g.*, analysis at high temperatures might be destructive for the low-stability oxyli-

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pins [14]. Our objective was to develop a simple reversed-phase (RP)-LC-MS/MS method for the identification of oxylipins in whole grains of wheat (*Triticum aestivum*), as the first study reported to our knowledge. The resulting LC-MS/MS chromatograms are statistically evaluated by variety-based comparison of peak heights by principal component analysis (PCA).

2 Experimental

2.1 Materials

Grains of eight different varieties of wheat were obtained from the Jõgeva Plant Breeding Institute (Estonia). The varieties used were Azurite, Helle, Manu and Picolo as spring and Ada, Anthus, Bjorke and Compliment as winter varieties (all harvested in 2007).

All chemicals were of analytical reagent grade and used as received. Methanol and formic acid from Fluka (Buchs, Switzerland) were used for extraction and separation, respectively. Acetonitrile and methanol of ultra-gradient grade used in the chromatographic experiments were from Romil (Cambridge, UK). Deionized water was prepared by using a Milli-Q system from Millipore (Bedford, MA, USA). Standard oxylipins [9-HODE, 13-HODE, 9,10-dihydroxy-12-octadecenoic acid (9,10-DiHOME)] were purchased from Cayman Europe (Tallinn, Estonia).

2.2 Extraction procedure

The wheat grains were milled to a fine powder using an ordinary grinder. About 2.0 g of finely ground wheat was weighed and extracted with 10 mL methanol in an ultrasonic bath for 30 min. After sonication, the samples were centrifuged for 5 min (3000 rpm) and the liquid phases were filtered through 0.45-μm filters. The liquid phases were taken to dryness in a rotary evaporator and redissolved in 0.5 mL methanol and injected directly into the LC system.

2.3 LC-MS/MS analysis

Samples were analyzed using LC/electrospray ionization (ESI)-MS/MS in negative ionization mode on an 1100 Series LC/MSD Trap-XCT (Agilent Technologies, Santa Cruz, CA, USA). The ion trap was connected to an Agilent 1100 Series HPLC instrument consisting of an autosampler, a solvent membrane degasser, a binary pump and a column thermostat. The HPLC 2D ChemStation software with a ChemStation Spectral SW module was used both for process guidance and for the processing of the results.

The samples were separated on a Zorbax 300SB-C18 column (2.1 × 150 mm; 5 μm particle size; Agilent Technologies) with a guard column filled with the same type of sorbent. The column was eluted at 0.3 mL/min with a linear gradient from 0.1% aqueous formic acid (solvent A) and 5% acetonitrile (solvent B) to 30% B in 40 min, followed by an increase to 90% B over 15 min. The column temperature was maintained at 35 °C and the sample injection volume was 15 μL. All experiments were performed in triplicate.

The conditions of MS/MS detection: m/z interval, 100–1000 amu; target mass, 400 amu; number of fragmented ions, 2; maximal collection time, 100 ms; compound stability, 100%; drying gas (N_2 from generator) speed, 10 L/min; gas temperature, 350 °C; gas pressure, 30 psi; collision gas (He) pressure, 6×10^{-6} mbar.

2.4 Principal component analysis

PCA was carried out using the Matlab (Mathworks, Natick, MA, USA) environment by standard singular value decomposition procedure. For data preprocessing, peak intensities were replaced by their logarithms (to reduce influence of large peaks) and mean centered. In PCA, a data matrix, d, is decomposed to the scores matrix t and loading matrix p as follows: $d = tp'$, where ' denotes transpose. In this decomposition, the d matrix contains LC-MS/MS data points in new, principal component axes, and the t matrix contains principal component coordinates.

3 Results and discussion

3.1 Selection of extraction conditions

It is supposed that extraction is a fundamental point in any analytical method. The extraction conditions must be adapted to the type of compounds finally identified. In this study, the extraction conditions were selected on the basis of the polarity of oxylipins. Using methanol as extraction solvent allowed us to perform the extraction in a reproducible way and to guarantee good stability of the extracts. As the primary goal of this work was to provide qualitative information about oxylipins in wheat, the extraction was performed only once.

3.2 Identification of oxylipins

All the oxylipins identified (Fig. 1, peaks 1–10) are various (per)oxidation products of linoleic (*cis*,*cis*-9,12-octadecadienoic) acid, most of them are characterized by the MS^2/MS daughter ion $-OOC(CH_2)_7CH\text{-}OH$ with m/z = 171 amu, containing a carboxyl and an OH-group. Neutral loss of 100 amu (*e.g.* 329–229; 295–195; 311–211) corresponds to the loss of the end-group $HO\text{-}CH{=}CH(CH_2)_3CH_3$ from an oxylipin molecule. Between these groups, for example, the fatty acid with $[M–H]^-$ = 329 (Fig. 2B) has a moiety $–CH_2CH(OH)\text{-}CH_2$- that corresponds to the neutral loss of 58 amu (229–171). The molecular structures of the com-

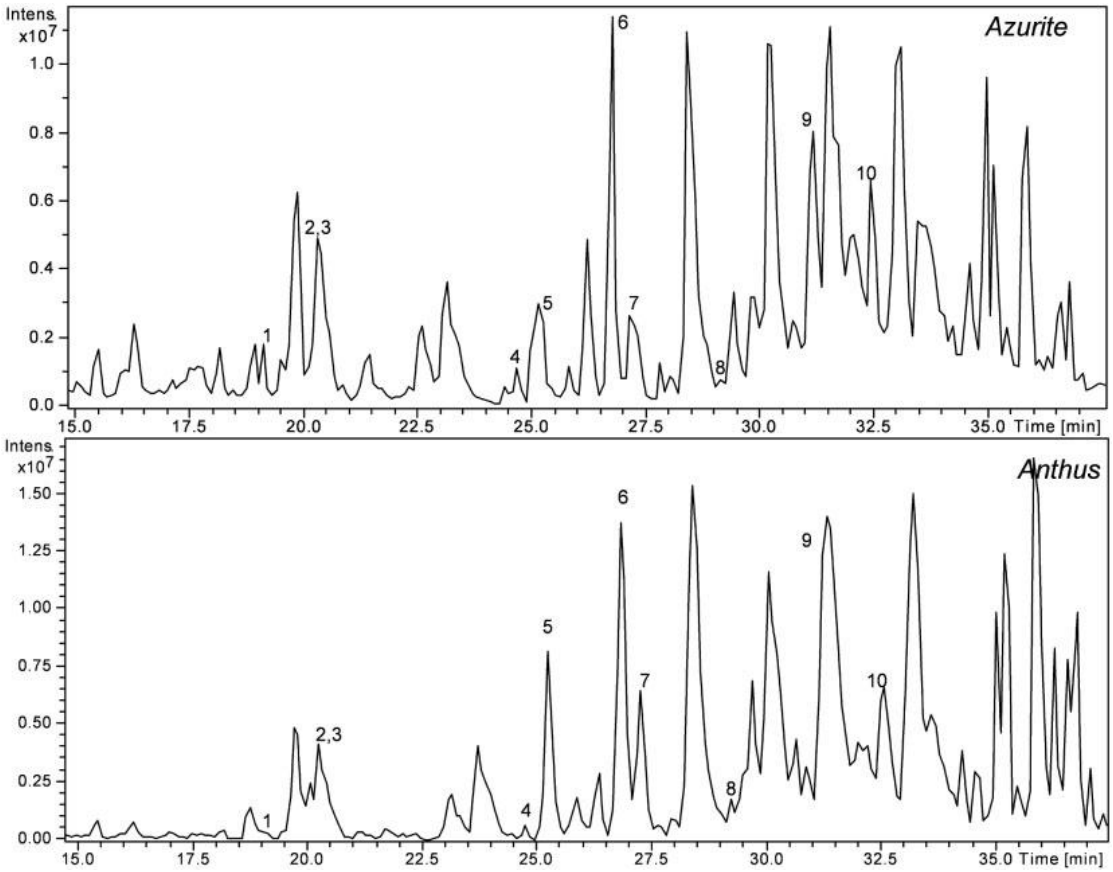


Figure 1. LC/ESI-MS/MS base peak chromatograms of the methanol extracts of Azurite and Anthus, a spring and a winter wheat variety, respectively. Peak numbers indicate the tentative oxylipins detected: (1) mixture of 9-oxo-12,13-dihydroxy-10-octadecenoic (9-oxo-DHODE) and 13-oxo-9,10-dihydroxy-11-octadecenoic (13-oxo-DHODE) acids ($[M–H]^-$ = 327); (2) 9,10,18-trihydroxy-octadecanoic acid (9,10,18-THODE) ($[M–H]^-$ = 331); (3) 9,12,13-trihydroxy-10-octadecenoic (9,12,13-THODE) acid ($[M–H]^-$ = 329); (4) 9-hydroperoxy-10,12,15-octadecatrienoic acid ($[M–H]^-$ = 309); (5) mixture of 13-hydroxy-9-oxo-10-octadecenoic (9-oxo-13-HODE) and 13-oxo-9-hydroxy-10-octadecenoic (13-oxo-9-HODE) acids ($[M–H]^-$ = 311); (6) 12,13-dihydroxy-9-octadecenoic acid (12, 13-DiHOME, iso-LTX-diol ($[M–H]^-$ = 313); (7) 9,10-dihydroxy-12-octadecenoic acid (9,10-DiHOME, LTX-diol) ($[M–H]^-$ = 313); (8) 15,16-epoxy-12,15-octadidecenoic acid (15,16-EODE) ($[M–H]^-$ = 293); (9) mixture of 13-hydroxy-9,11-octadecadienoic (13-HODE) and 9-hydroxy-10,12-octadecadienoic (9-HODE) acids ($[M–H]^-$ = 295); (10) 9,10-epoxyoctadecanoic acid (9,10-EODA) ($[M–H]^-$ = 297).

pounds were verified either by comparison of the daughter ion spectra with the respective spectra of standard fatty acids (9-HODE, 13-HODE, 9,10-DiHOME) or with the respective spectra from the literature [20–24].

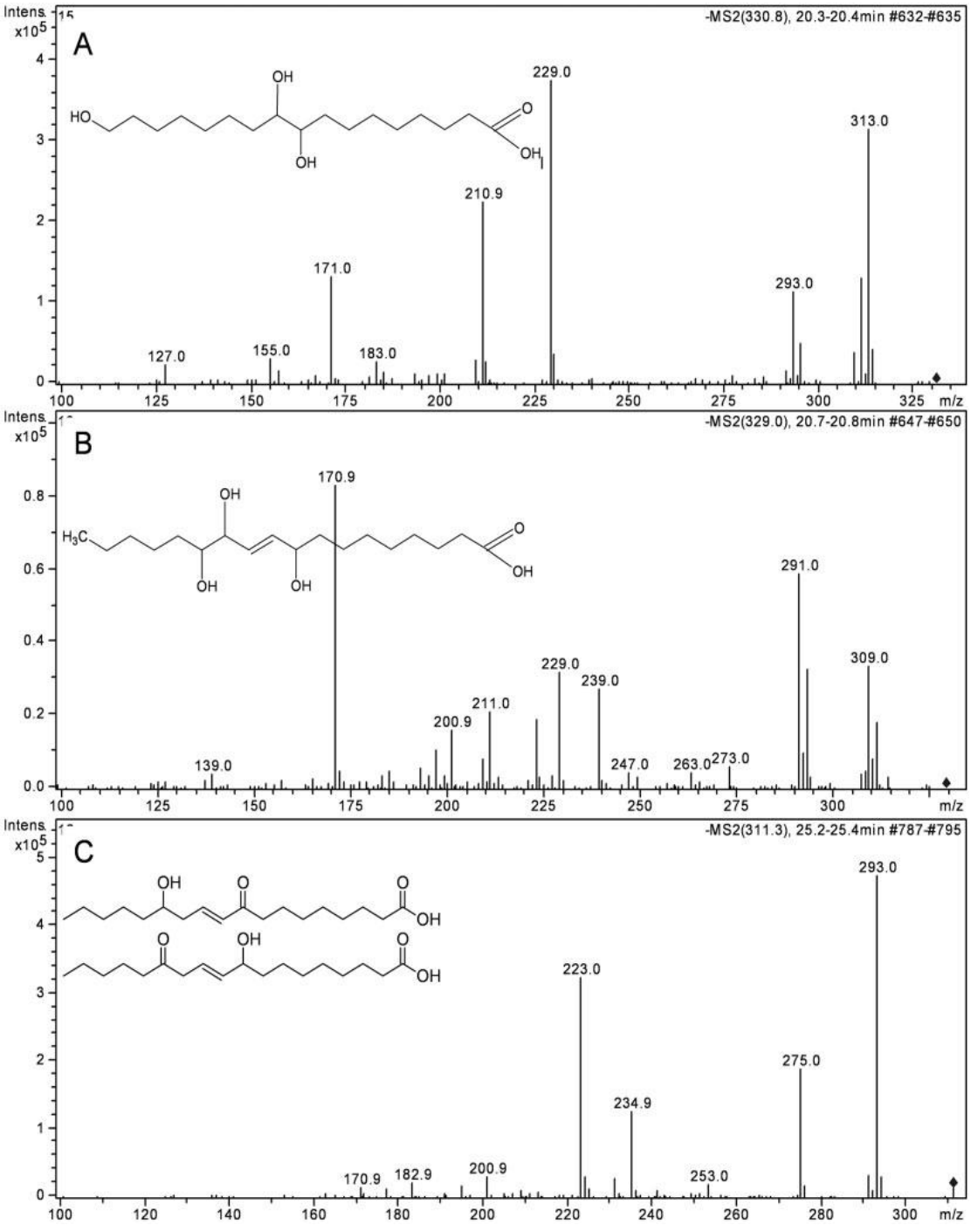
In some cases, chromatographically unresolvable mixtures of positional isomers as well as stereoisomers of an oxylipin were formed. For example, one of the highest peaks in the base peak chromatogram is characterized by a pseudomolecular ion $[M–H]^-$ = 295, representing a mixture of two HODE (9-HODE and 13-HODE) (Fig. 1, peak 9).

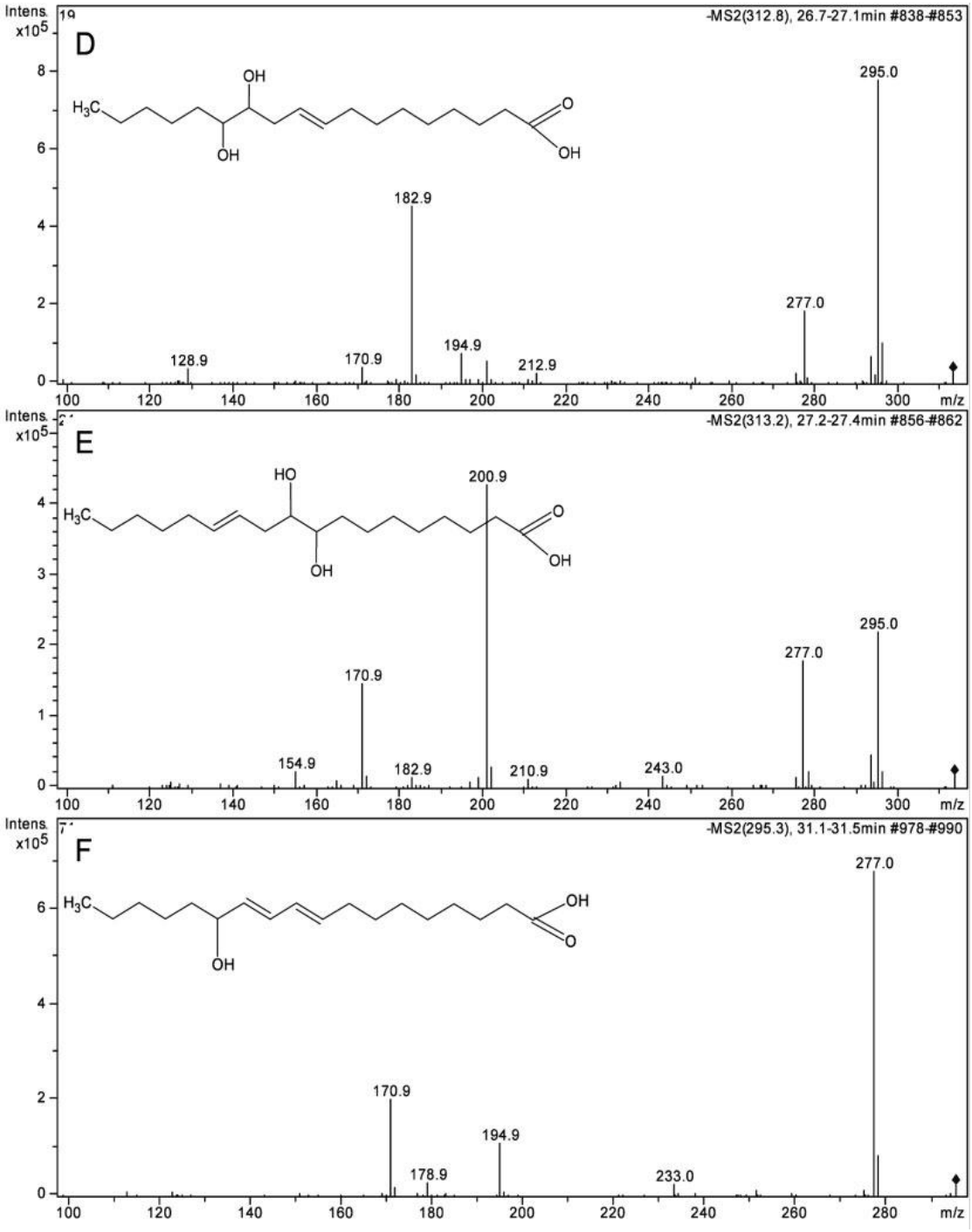
Fatty acid negative ions with $[M–H]^-$ = 293, 297, 309, 313, 327, 331, all possessing the characteristic negative daughter ion 171, were also discovered.

Further investigations should clarify the complicated oxidation scheme of PUFA producing all these oxylipins.

3.3 Comparison of different wheat varieties

To compare different varieties of wheat, the data as a whole were next subjected to PCA. Data analysis was carried out as





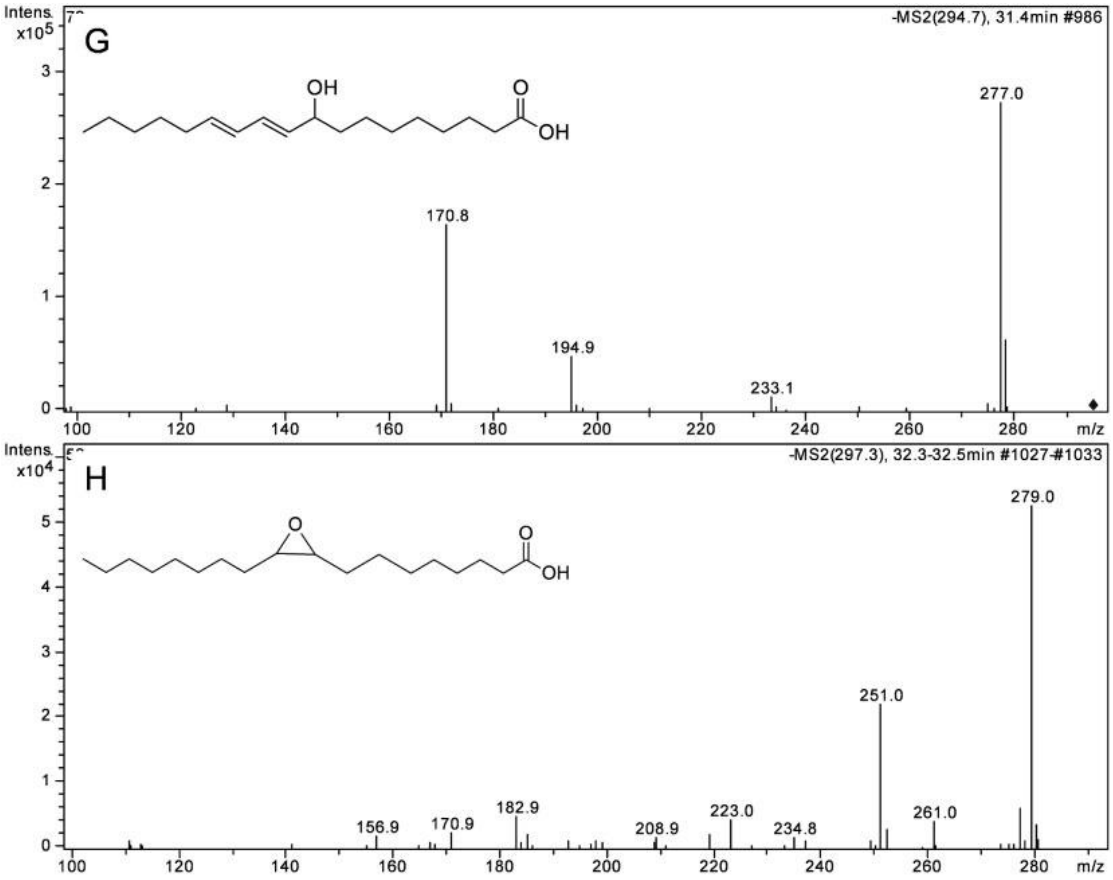


Figure 2. MS^2/MS spectra of the most abundant oxylipins identified in wheat: (A) 9,10,18-THODE; (B) 9,12,13-THODE; (C) mixture of 9-oxo-13-HODE and 13-oxo-9-HODE; (D) 12, 13-DiHOME (iso-LTX-diol); (E) 9,10-DiHOME (LTX-diol); (F) 13-HODE; (G) 9-HODE; (H) 9,10-EODA. For full names of the compounds see Fig. 1.

described in Materials and methods. In Fig. 3, LC-MS/MS data are represented in two first principal component coordinates. Each point represents one particular chromatogram. As can be seen in Fig. 3, the wheat varieties Ada, Manu, Picolo and Bjorke are nicely separated using this approach. To find out what feature (m/z) is responsible for the separation of samples (*i.e.* sorting), loading vectors located at columns of matrix p are introduced and represented in Fig. 3. It is evident from Fig. 3 that vectors corresponding to the m/z values 297, 311 and 329 are the main responsible ones for the separation of the above-mentioned wheat varieties. Also, from Fig. 3, spring and winter wheat varieties as two standalone groups can be evaluated. An area of striated rectangle in Fig. 3 denotes the spring wheat varieties. Those varieties that belong to either the spring or the winter group have more group-specific features and are bunched together with one exception: Anthus. Still, more grain samples of different varieties must be analyzed to confirm this point.

3.4 Possible adverse physiological effects of the oxylipins

The health deteriorating effect of oxidized fats has been known for decades [25], but mostly it has not been associated with concrete oxidation products. One of the few exceptions is 9,10-DiHOME ($[M–H]^-$ = 313; peak 7 in Fig. 1), known also as leukotoxin diol (LTX-diol) and its isomer 12,13-dihydroxy-9-octadecenoic acid or isoleukotoxin-diol (iso-LTX-diol; peak 6 in Fig. 1), which were discovered by us in wheat grains. The structure of these compounds was proved by

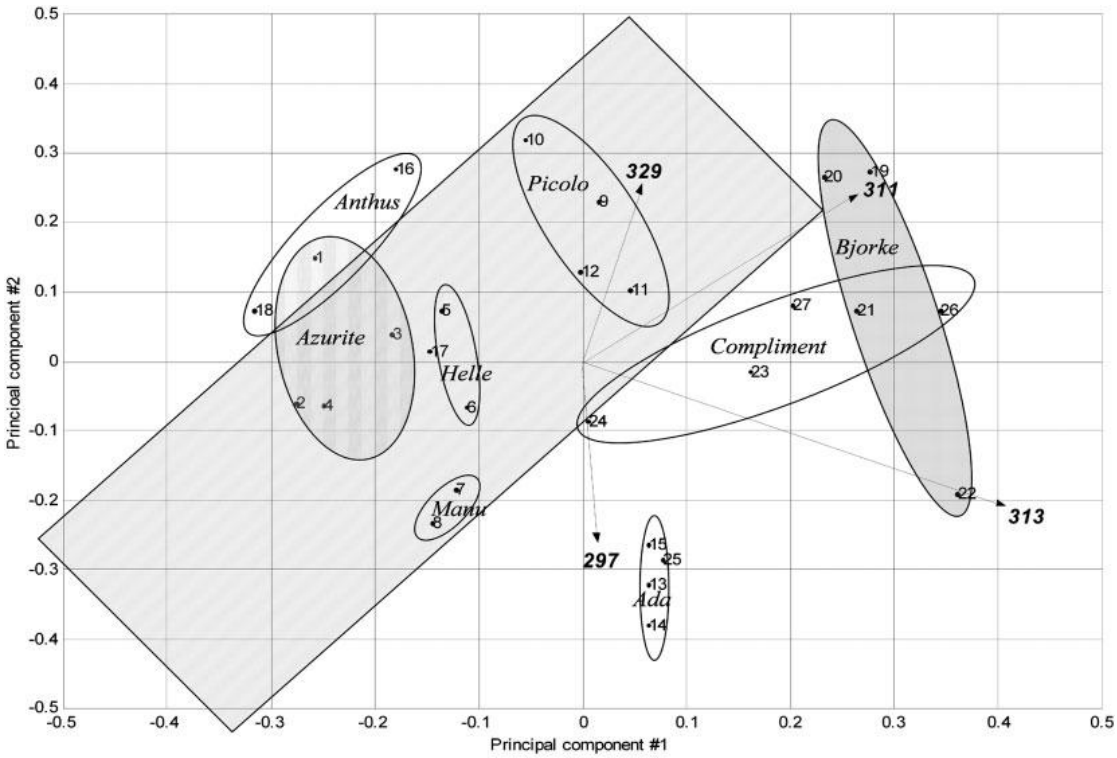


Figure 3. Representation of the LC-MS/MS chromatograms in the first principal component coordinates. Each point represents one particular chromatogram. Vectors are loadings and the numbers 297, 311, 313 and 329 refer to *m/z* values that primarily are responsible for the scattering. The striated rectangle indicates the spring wheat varieties.

comparison of their MS^2/MS daughter ion spectra (Fig. 2E) with both the spectra reported in the literature [22] and with the fragmentation spectra of the respective commercial standards. All these fragmentation spectra had a very good congruence.

The acute toxicity of the endogenous LTX-diols is well characterized [26, 27]. Furthermore, LTX-diols exerted also mitogenic activity and stimulated human breast cancer cell proliferation *in vitro* [28]. It was recently found that exogenous LTX-diols act additively to disrupt the endocrine function in female rats [29].

Whether the LTX-diols in wheat grains have any real toxic consequence for human organisms, especially after baking, needs further investigations.

4 Conclusions

In this work, it is demonstrated that oxylipins, products of free fatty acid peroxidation, can be identified in wheat grains by a relatively simple LC-MS/MS method using RP chromatographic columns and an aqueous formic acid/acetonitrile gradient as mobile phase. It can be assumed that the identified oxylipins in the different wheat varieties are variety specific, involving differences in their quantities rather than in the nature or presence/absence of some of these compounds. In this work, several different oxylipins were identified, and it is suggested that, for example, 12,13-dihydroxy-9-octadecenoic acid (iso-LTX-diol), characterized in all of the grain samples by the highest oxylipin peak, could be a good candidate for a marker of lipid oxidation in the grains (Fig. 1, peak 6). Further investigations, including analysis after time intervals within the same harvest, are necessary to confirm this statement.

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Conflict of interest statement

The authors have declared no conflict of interest.

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ARTICLE III

M. Vaher, K. Matso, T. Levandi, K. Helmja, M. Kaljurand. Phenolic compounds and the antioxidant activity of the bran, flour and whole grain of different wheat varieties. Procedia Chemistry. 2010, 1, 76-82.

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Phenolic compounds and the antioxidant activity of the bran, flour and whole grain of different wheat varieties

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Abstract

Total phenolic content and DPPH radical scavenging capability of the bran layer, flour made from endosperm and whole grain of wheat were determined. Fifteen different wheat samples of ten spring and five winter wheat varieties were analyzed. The spring wheat varieties were grown in both conventional and organic conditions. The total phenolic content of the bran layer found to be the highest (1258-3157 μg/g), followed by that of grains (168 - 459 μg/g) and the lowest of flour (44 - 140 μg/g). The bound phenolic acids were quantified by CE-DAD analysis after alkaline hydrolysis. Ferulic acid was a major compound among phenolic acids found in wheat varieties.

Keywords: Wheat, phenolics, antioxidant activity, capillary electrophoresis, DPPH

1. Introduction

Wheat (*Triticum aestivum)* is an important agricultural commodity and a primary food ingredient worldwide. It contains important beneficial nutritional components. Wheat and wheat-based food ingredients rich in natural antioxidants can ideally serve as a basis for development of the functional foods designed to improve the health of millions of consumers. Growing evidence indicates that intake of whole wheat foods may associated with health benefits including the reduced risk of coronary heart diseases and certain type of cancers [1,2]. These beneficial effects are attributed to the bioactive factors in wheat grain such as non digestible carbohydrates and phytochemicals [1-3]. One group of phytochemicals with small molecular weight present in wheat grain is antioxidants. These include but are not limited to carotenoids, tocopherols, lignans, flavonoids and phenolic acids. Antioxidants are defined as molecules that, at low concentration and specific assay conditions, can delay or prevent oxidation of an oxidizable substrate [4]. These antioxidative components may prevent life important molecules such as DNA and enzymes from oxidative damages through different mechanisms. For instance, wheat antioxidants may directly react with the reactive oxygen species (ROS), such as hydroxyl radicals or singlet oxygen molecules, to terminate the attack of the latter on biological molecules.

There are a number of studies on the phenolic acids contained in wheat [5-8], while information on the content of the other polyphenols (lignans, flavonoids) of wheat is poor. Most lignans were found in wheat bran [9-11]. Several flavones (apigenin, luteolin, chrysoeriol, tricin) were detected in the leaf tissue of wheat in their glycoside forms

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[12]. The major flavonoids in wheat are apigenin-C-diglycosides (flavonols) [13], cyanidin-3-glycosides and peonidin3-glycoside (anthocyanins) [14].

Phenolic acids and flavonoids are present in cereals in free, soluble conjugated and insoluble bound forms. Previous studies reported on the presence of phenolic acids in wheat grains mostly in the bound form, while the majority of them exist in the aleurone layer and bran associated with cell wall materials, such as polysaccharides and lignans [15-18].

HPLC has mainly been used to determine the phenolic content of wheat [13,19-20] and only some authors have separated phenolic acids by capillary electrophoresis [21].

The aim of the present work was to compare the antioxidant activities of different wheat varieties and determine their total phenolic content spectrophotometrically. For the analysis of bound phenolic acids as the most abundant form of phenolic acids in wheat, capillary electrophoresis (CE) was employed. The main advantages of the latter are high efficiency, reduced sample and solvent requirements and simple instrumentation.

2. Experimental

2.1. Materials

Wheat grains and their respective bran and flour of different varieties, which were grown in both conventional and organic conditions, were obtained from Jõgeva Plant Breeding Institute (Estonia). The winter varieties analysed were Anthus, Bjorke, Olivin, Portal, Tarso while of the spring varieties, Helle, Manu, Meri, Triso, Vinjett were subjected to analysis. All samples were harvested in 2008 (Table 1).

All the chemicals were of analytical grade and were used as received. For extraction, methanol from Fluka (Buchs, Switzerland) and diethyl ether from Sigma–Aldrich (Germany) were used. Sodium tetraborate, sodium hydroxide, sodium sulfate anhydrous, hydrochloric acid, Folin-Ciocalteau reagent and standard phenolic acids (syringic, vanillic, ferulic, p-coumaric, caffeic, gallic and sinapic acids) were purchased from Sigma-Aldrich (Germany).

Table 1. The wheat varieties investigated

Sample no	Variety	Winter/spring variety	Growth conditions
2	Helle	spring	conventional
3	Manu	spring	conventional
4	Meri	spring	conventional
12	Triso	spring	conventional
13	Vinjett	spring	conventional
17	Anthus	winter	conventional
18	Bjorke	winter	conventional
26	Olivin	winter	conventional
28	Portal	winter	conventional
31	Tarso	winter	conventional
35	Helle	spring	organic
37	Manu	spring	organic
38	Meri	spring	organic
44	Triso	spring	organic
45	Vinjett	spring	organic

2.2. Extraction of free phenolics

Wheat grains were milled to a fine powder by using an ordinary grinder. The bran layer and flour were used as received. Three replicates (2 g each) of wheat bran layer, flour and grain were individually mixed and sonicated with methanol for 30 min. After sonication, the samples were centrifuged for 10 min at 2500g. The extraction procedure was repeated twice, the supernatants were pooled and evaporated to dryness and redissolved in 0.5 ml of methanol. The samples prepared were stored in a freezer until further analysis within a 3-month period.

2.3. Alkaline hydrolysis

After the methanol extraction the residue was hydrolyzed with sodium hydroxide. 20 ml of a 2M NaOH were mixed with 1 g of wheat bran layer, flour and grain residue. The mixture was mixed for 4 h and the pH was adjusted to two with a 6M HCl. Diethyl ether (100 ml) was added to the mixture, the container was inverted 15 times and then centrifuged at 1000g for 10 min. The supernatant was removed and the process was repeated with 75 ml of diethyl ether. The supernatants were pooled, dried with anhydrous Na_2SO_4, evaporated to dryness and redissolved in 0.5 ml of methanol.

2.4. Determination of total phenolic content

Each fraction of free phenolics (0.2 ml) was mixed with 1 ml of the Folin-Ciocalteau reagent and 0.8 ml of a saturated sodium carbonate solution. The mixture was allowed to stand at room temperature for 30 min and then the absorbance was measured at 765 nm in a Varian Cary 3C spectrophotometer (Varian analytical instruments, Harbor City, CA). The total phenolic content was expressed as microgram of gallic acid equivalent (GAE) per milliliter of solution. From these data the total phenolic contents of wheat bran, grain and flour were subsequently calculated.

2.5. Assay of DPPH radical scavenging activity

The DPPH free radical scavenging activity of wheat bran extracts was determined using a1mM DPPH solution. Each sample of wheat extracts at different concentrations in methanol (2 ml) was mixed with 2 ml of a methanolic solution containing 1 mM DPPH. The mixture was shaken, and then left to stand for 30 min in the dark. The absorbance was measured at 517 nm. The absorbance of the control was obtained by replacing the sample with methanol. The DPPH radical scavenging activity of the sample was calculated as follows:

DPPH radical scavenging activity (%) = [1- absorbance of sample/absorbance of control] x 100.

All the tests were performed in triplicate. The EC_{50} value was determined to be an effective concentration at which the DPPH radical was scavenged by 50%. The EC_{50} value was obtained by interpolation from a linear regression analysis.

2.6. CE analysis

All experiments were performed using an Agilent System (Agilent Technologies, Waldbronn, Germany) with a UV-Vis diode array detector (DAD). A CE Chemstation was used for instrument control and data handling. The separation of phenolic compounds was performed in a fused silica capillary (Polymicro Technology, Phoenix, AZ, USA) with 50 μm i.d., 60 cm total length and 52 cm effective length. All measurements were carried out at 25˚C. The diode array detection was used over the range of 190-600 nm to obtain spectral data. Detection took place at 210 nm. Peak identification was achieved by comparing both the migration time and spectral data obtained from real samples and standards. In order to assure the identification of the selected compounds, the real samples were spiked with increasing amounts of each standard.

The calibration graphs were obtained by plotting the concentration (μg/ml) of selected phenolic acids in the range of 10-100 μg/ml, against peak areas, showing good correlation coefficients (≥ 0.989) for all compounds.

Prior to use, the capillary was rinsed with a 0.1 M NaOH solution for 5 min and with the separation buffer for 5 min. A 50 mM sodium tetraborate (pH 9.3) as a separation buffer was used. The applied voltage for the separation of polyphenols was + 20 kV.

3. Results and discussion

3.1. Free phenolics and antioxidative activity

The free phenolics of fifteen wheat samples were extracted separately from the bran layer, flour and grain (Table 1) with methanol in an ultrasonic bath. The phenolic contents (μg GAE/g) were determined by the Folin-Ciocalteau assay, the results are presented in Fig. 1.

It is evident from Fig. 1., that the bran layers have the highest content of total phenolics ranging from 1258 to 3157 μg GAE/g. This is not surprising because it is well known that phenolic compounds are concentrated in the bran and germ fractions of wheat that are removed during the milling of wheat into white flour. A comparison of the total phenolic contents of spring and winter wheat varieties, showed the latter to be more stable (samples 17, 18, 26, 28 and 31) in contrast with conventional spring varieties (samples 2, 3, 4, 12 and 13), whose content of total phenolics differed a lot and therefore may be assumed to be more variety-specific. Highly significant differences in total phenolic content were detected between the spring wheat variety Manu (1258 μg GAE/g) and Triso (3157 μg GAE/g). A similar trend may be observed in the case of whole grain and flour. Literature data report on different total phenolic contents of different parts of wheat grain. Use of different standards for the measurements of total phenolic contents makes the respective comparison quite difficult. Moore et al. [6] measured the total phenolic content of the bran of 20 different wheat genotypes. The phenolic content ranged from 2700 to 3500 μg GAE/g. Yu et al. [23] reported the content of phenolics of wheat flour to be from 177 to 257 μg GAE/g. These values are good comparable with our measurements. The researchers also found total phenolic content is closely associated on wheat genotype [6,22].

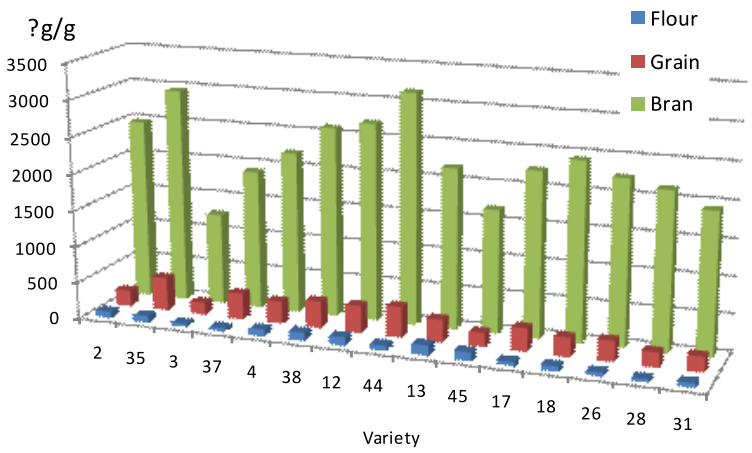

Fig. 1. The total phenolic content of the bran layer, flour and whole grain of different spring and winter varieties determined by the Folin-Ciocalteau assay. The results are presented as microgram gallic acid equivalents (GAE) per gram of wheat samples.

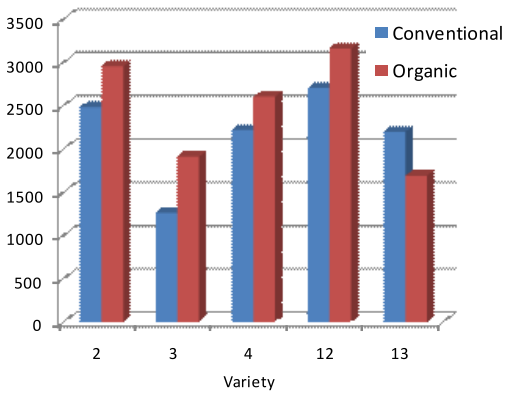

Fig. 2. Total phenolics of the spring wheat varieties grown in different conditions.

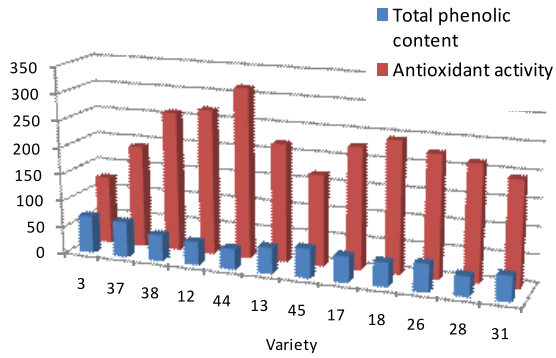

Fig. 3. The total phenolic content (mg GAE /100g (red)) and antioxidant activity EC_{50} (blue) of the bran layer of selected wheat varieties.

As seen from Fig. 2. the total phenolic content of the spring wheat varieties grown in organic conditions is a little higher than that of the wheat grown in conventional conditions, with one exception only. From these results it may be concluded that growing conditions have a certain effect on the biosynthesis and accumulation of phenolic compounds.

Phenolic compounds have a potent antioxidant activity, their total phenolic content has been found to be significantly associated with different measure of antioxidant activity [23] including DPPH scavenging capacity [7]. It can be seen from Fig. 3 that the extracts with a higher content of phenolics possess a higher antioxidant activity (lower value of EC_{50}). The difference in antioxidant activity between different wheat varieties may be due to the different composition of the phenolic compounds present.

3.2. CE analysis of bound phenolic acids

From literature it is known that the phenolic acids in wheat grains are present mostly in the bound form with other grain components such as starch, cellulose, β-glucan and pentosane [15,24]. Insoluble bound phenolics may be released by the base, acid or enzymatic treatment of samples prior to extraction. In the present work, the alkaline hydrolysis was performed before extracting these compounds with diethyl ether.

To determine the concentration of each individual phenolic acid, CE was used for separation and each fraction was subsequently quantified by UV adsorption. As shown in Fig. 4, six phenolic acids were separated and identified. This method is well reproducible and provides good separation in terms of migration time and resolution.

The results of quantification are presented in Table 2. CE analysis showed that in the extracts of bound phenolic acids of two wheat varieties, a dominant phenolic acid identified was ferulic acid, followed by sinapic, syringic, vanillic and p-coumaric acids. The caffeic acid was present only in the spring variety. The content of ferulic and sinapic acids of the winter variety was two times higher than that of the spring variety, respectively 532.4 μg/g and 268.9 μg/g for ferulic and 272 μg/g.1 and 121.1 μg/g for sinapic acid. Ferulic acid comprise of 48% and 60% among total phenolic acids in spring and winter wheat bran, respectively.

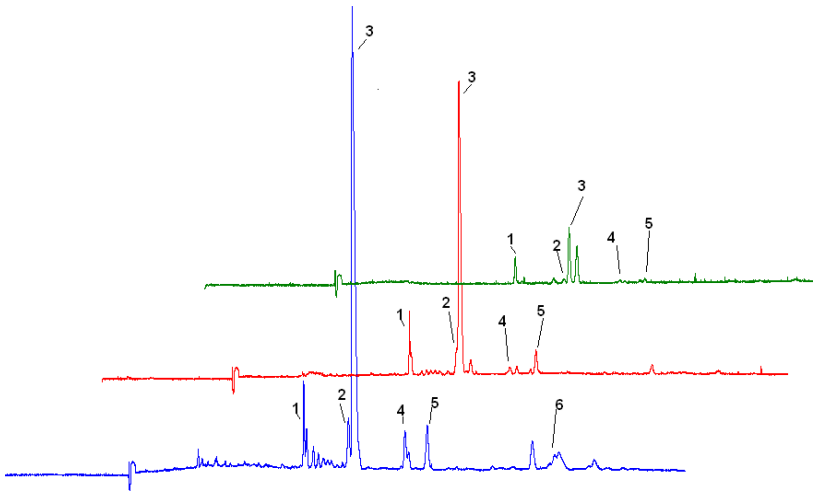

Fig. 4. The electropherograms of bran (a blue line), whole grain (a red line) and flour (a green line) extracts of the spring wheat Helle. Separation buffer: 50 mM sodium tetraborate (pH 9.3). The phenolic acids separated: 1 – sinapic acid, 2- syringic acid, 3 – ferulic acid, 4 – p-coumaric acid, 5 – vanillic acid, 6 - caffeic acid.

From Table 2 it is seen that the total content of bound phenolic acids of the winter wheat variety (892.2 μg/g) is higher than that of the spring variety (569.1 μg/g). It may be assumed that in the case of winter wheat the environmental conditions in the growing phase are more stressful for the plant. This is an agreement with well know fact that phenolic compounds as secondary metabolites are synthesized by plants during growing phase more in response to stress than in normal conditions.

Table 2. The phenolic acids content of different wheat samples

	Phenolic acids, μg/g					
	Caffeic acid	Ferulic acid	p-Coumaric acid	Syringic acid	Vanillic acid	Sinapic acid
			Spring wheat			
Bran	107±9	268.9±49	18.5±1.7	24.4±2.1	29.1±2.7	121.1±11.8
Grain	-	132.8±10	2.8±0.2	13.1±1.2	24±2.1	87.3±2.7
Flour	-	14.4±1.5	0.7±0.05	9.8±0.8	21.1±2.0	58.1±1.7
			Winter wheat			
Bran	-	532.4±52.6	17.7±1.4	37.9±3.2	32.1±2.8	272.1±4.7
Grain	-	154±14.7	4.2±0.3	20.9±0.8	23.3±2.4	182.3±4.4

4. Conclusions

It was demonstrated that CE is a suitable and accurate method for the separation and quantification of phenolic compounds in wheat samples. The content of bound phenolic acids of winter wheat was found to be significantly higher than that of spring wheat. The contents of total free phenolics of all winter wheat varieties were quite similar. It was also established that the phenolic content of the wheat varieties grown in organic conditions grown was higher than that of the wheat varieties grown in conventional conditions.

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ARTICLE IV

T. Levandi, T. Püssa, M. Vaher, A. Ingver, R. Koppel, M. Kaljurand. Principal component analysis of HPLC-MS/MS patterns of wheat (*Triticum aestivum*) varieties extracts. (*accepted to the Proceedings of the Estonian Academy of Sciences*)

Principal component analysis of HPLC-MS/MS patterns of wheat (*Triticum aestivum*) varieties extracts

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Abstract

Untargeted metabolomic strategy was chosen to investigate “as-many-small metabolites as-possible” in a collection of 13 varieties of spring, winter and organic wheat (*Triticum aestivum*). Metabolites were separated by high-performance liquid chromatography on a reversed-phase column (RP-HPLC) coupled with electrospray ionization tandem mass-spectrometry (ESI-MS/MS). The procedure includes extraction of metabolites, chromatographic separation using linear gradient of aqueous formic acid and acetonitrile with subsequent identification of compounds by MS/MS. Discrimination of the metabolomic patterns of different wheat varieties was achieved by the use of principal component analysis (PCA). Results of PCA indicate clear differences in the patterns of wheat varieties.

The winter wheat, grown in conventional conditions and the spring wheat, grown in organic conditions differ from spring wheat grown in conventional conditions by the higher level of carbohydrates content. It could be explained by an exposure of osmotic stress resistance. Varieties grown under organic conditions are well distinguished from others by the results of PCA , which point to the existence of an impact of different farming systems.

Keywords

HPLC-MS/MS, PCA, wheat varieties, plant phenolics

INTRODUCTION

Common wheat (*Triticum aestivum* L.) is a very diverse and widely adaptable cereal crop. Respective breeding programs have been primarily targeted at selection of new cultivars with higher grain yields and end-use quality. During the last decade, need for increasing sustainability and environmental protection have become more relevant in the agricultural sector. For that reason, special cultivars of wheat for organic farming are becoming a challenge for breeders [1-3].

In wheat, as in the other plants, antioxidant compounds are naturally synthesized as a part of multifunctional defense systems against the detrimental effects of (per)oxidation. It is apparent that selecting and breeding wheat genotypes that are rich in antioxidants will improve agronomical traits of the wheat plants, enhance the keeping quality, stability, and safety of wheat products and improve the health benefits associated with wheat consumption [2]. It is reported [4-5] that variation in the antioxidant activity in terms of DPPH scavenging capacity, total phenolic content, and concentrations of phenolic acids likely indicates significance of the genotype effects on the antioxidant properties of whole wheat and wheat fractions including bran. The patterns of variation of phenolic acid concentrations have been dissimilar among genotypes [4,6]. Significant variation in the ferulic acid (a predominant phenolic acid in the grains of all of the tested soft wheat varieties or lines) concentration of wheat genotypes has been found to be correlated with the disease resistance [2].

Organic agriculture is gaining popularity and needs a variety of improvements for further optimization of respective farming system. Organic agriculture is focused on varieties with ecologically better-adapted traits, which should yield without use of fertilizers and

pesticides, being at the same time oriented in high quality production. Several reports deal with outcomes of organic field trials, organic plant breeding and crop production [1,3,7].

Metabolomic approaches have emerged as a valuable tool for the plant sciences, including the study of development, the phenotyping of genetically altered plants, qualitative trait analysis, and the improvement of breeding strategies. In addition, metabolomic technologies can be utilized for the discovery and identification of markers of diseased and stressed plants, as well as changes following genetic modifications and characterization of different genotypes/phenotypes. In the past few years, rapid development of high-throughput tools for metabolic profiling, such as detection of the levels of multiple metabolites in a single extract, have facilitated the analysis of a broad range of metabolites. It contributes to a greater understanding of the metabolism network and the mechanism of its interaction with developmental phenotypes [8,9].

The most common separation and detection techniques for the profiling of metabolites are liquid chromatography (LC) in its high performance (HPLC) or ultra performance (UPLC) forms; gas chromatography (GC), capillary electrophoresis (CE), as well as the coupling of these instruments with detection techniques such as mass spectrometry (MS) [10-15] and nuclear magnetic resonance (NMR)[16,17]. Different techniques used in metabolomic analysis are described in several reviews [8,9,18-20].

The objective of this research was to investigate that composition of wheat extracts which contributes to the characterization of wheat varieties through the comparative profiling of metabolites found in different wheat varieties. Reversed-phase (RP)-LC-MS/MS method for separation and identification of metabolites in the whole grains, bran and flour of wheat was developed for that purpose.

Resulting LC-MS/MS chromatograms were statistically evaluated by a variety based comparison of peak heights using principal component analysis (PCA) of a whole data set.

EXPERIMENAL

Materials

Grains, flour, and wheat bran were obtained from the Jõgeva Plant Breeding Institute (Estonia). The varieties investigated were Manu, Meri, Triso, Vinjett, (items 1-4) and Spelta (*Trticum spelta*, item 5) as spring varieties, Anthus, Bjorke, Olivin, Portal, and Tarso (items 6-10) as winter varieties, and Manu, Meri, and Vinjett (items 11-13) as spring organic varieties (all harvested in 2009).

All chemicals were of analytical grade and used as received. Methanol and formic acid from Fluka (Buchs, Switzerland) were used for extraction and separation, accordingly. Acetonitrile and methanol of ultra gradient grade used in the chromatographic experiments were from Romil (Cambridge, UK). Deionized water was prepared by a Milli-Q system from Millipore (Bedford, MA, USA).

Extraction procedure

It is supposed that the extraction method is of fundamental importance for any analysis. Ordinarily, the extraction conditions must be adapted to the type of compounds finally identified. While this study was aimed as untargeted, methanol was used as an extraction solvent according to the results of our previous work [10] which allowed the performance

of better extraction in a reproducible way and guaranteed the high stability of extracts compared with methanol/water mixture in ratio 50:50. As the primary goal of this work was to provide qualitative information about metabolites in wheat, the extraction was performed only once.

The extraction procedure was as follows: the wheat grains were ground to a fine powder using an ordinary grinder; the bran layer and flour were used as received. About 2.0 g of finely ground wheat was weighed and extracted with 10 ml of methanol in an ultrasonic bath at 36 °C for 30 min. After sonication, samples were centrifuged for 5 min (3000 rpm), and liquid phases were filtered through 0.45 μm filter. Liquid phases were taken to dryness in a rotary evaporator, re-dissolved in 0.5 ml of methanol and injected directly into the LC system.

To obtain more hydrophilic extraction conditions, a methanol/water mixture in ratio 50:50 was used as a solvent a similar extraction procedure. The analytical samples obtained were stored at -18 °C.

LC-MS/MS analysis

Samples were analyzed using LC/ESI-MS/MS in the negative ion mode on an 1100 Series LC/MSD Trap-XCT (Agilent Technologies, Santa Cruz, CA, U.S.A.). The ion trap was connected to an Agilent 1100 Series HPLC instrument consisting of an autosampler, solvent membrane degasser, binary pump and column thermostat. The HPLC 2D ChemStation software with a ChemStation Spectral SW module was used both for process guidance and for the processing of the results.

The sample components were separated on a Zorbax 300SB-C18 column (2.1×150 mm; 5μm particle size; Agilent, Santa Cruz, CA, U.S.A) with a guard column filled with the same type of sorbent. The column was eluted at 0.3 ml/min with a linear gradient from 0.1% aqueous formic acid (solvent A) and 5% of acetonitrile (solvent B) to 30% B in 40 minutes followed by 90% B for 15 minutes. The column temperature was maintained at 35 °C and the sample injection volume was 15 μl. All experiments were performed in duplicate.

The conditions of MS/MS detection in the auto MS(n) regime with the scan mode standard enhanced were as follows: m/z linear spectra interval 100-1000 amu; target mass – 400 amu; number of precursor ions – 2; maximal collection time – 100 ms with 15 averages; compound stability – 100 %; drying gas (N_2 from generator) speed 10 l/min, gas temperature 350°C; gas pressure 30 psi, collision gas (He) pressure $6 \cdot 10^{-6}$ mbar.

Principal component analysis

PCA is a powerful tool for data analysis, identification of data patterns and expressing data, which enables the highlighting of a group's similarities and differences [21]. It can be assumed that the content and/or diversity of metabolites in the collection of varieties under investigation should be different. Winter and spring varieties are classified in terms of the growing season and are therefore considered as different phenotypes.

For PCA, the chromatograms of wheat varieties were transformed to a table (a matrix) of metabolites peak intensities. In this table, a row corresponds to a certain variety and a column to a metabolite (represented via a corresponding extracted ion peak intensity). If we

denote this matrix as, D, the PCA procedure decomposes the matrix D as follows: $D=ST^T$ (here superscript means transpose). Assuming that dimension of D is nxm, where n is a number of varieties under study and m is a number of measured peaks, the dimension of a scores matrix, S, is nxp and the dimension of the loadings matrix T, is mxp where $p<<n$. Plotting the first row of S versus its second row, a PCA plot is obtained where each point represents a variety. Moreover, if the first two components of T are overlayed onto the scores plot as vectors, the directions of these vectors explain the scatter and clustering of the varieties that are plotted on the scores plot.

PCA was carried out using a Matlab (Mathworks, Natick MA, USA) environment using a standard singular value decomposition procedure. For data processing, the peak intensities were replaced by their logarithms to reduce the influence of large and mean-centered peaks.

RESULTS AND DISCUSSIONS

Comparison of different wheat varieties

LC/ESI-MS/MS analysis of wheat extracts revealed the presence of different features. Twenty-three major features were detected in wheat grain extracts and almost fifty in the bran extracts.

To compare different varieties of wheat, the data as a whole were subjected to PCA after LC-MS/MS analysis. In Figure 1, the LC-MS/MS data are represented in two first principal component coordinates. Each point represents a peak height of a particular m/z value. As can be seen, this approach enabled good separation and grouping of the investigated varieties. To find out which feature (m/z) is responsible for the separation of samples (i.e.

sorting) loading vectors located columns of *p* matrix are also represented in Figure 1. It is evident from Figure 1 that vectors, corresponding to the m/z values 341 and 452, are those mainly responsible for the separation. Also, in Figure 1, organic, spring and winter varieties as three standalone groups can be distinguished. Those varieties that belong to the spring, winter, or organic groups have more common features and are bunching together, with two exceptions: Anthus (item 6) and Björk (item 7). According to our data, Anthus and Björk have lower gluten content. Anthus was also characterized as an exception in our previous work [22]. Still, more grain samples of different varieties must be analyzed to confirm this point.

It can be observed from Figure 1 that both the winter wheat grown in conventional conditions and the spring wheat grown in organic conditions differ from spring wheat grown in conventional conditions of the high level of content of various oligosaccharides. It can be explained with an exposure of osmotic stress resistance [23].

Flour and bran portions and the whole grains

The flour and bran portions of the grain and the whole grains of the same varieties were separately analyzed. Spring-Vinjett (item 4), organic-Vinjett (item 13) and winter-Bjorke (item 7) varieties were used for variety comparison.

In Figure 2, chromatograms of different fractions of different varieties are represented in PCA coordinates. It can be seen that vectors, corresponding to the m/z values 341 and 563, are those mainly responsible for the separation. It can also be observed from Figure 2 that only the bran fractions of the investigated varieties are significantly distinct from each

other. It may be assumed that differences between the bran fractions are strongly dependent on variety. It is widely accepted that wheat antioxidants and other beneficial phytochemicals (including phenolic compounds) are concentrated in the bran fraction of wheat grain [2].

Another observation that can be made in Figure 2 is that the organic variety may show characteristic behavior. It could be explained by the organic growing conditions. Nevertheless, substantially more samples of different varieties must be analyzed to confirm the last finding.

Identification of selected key metabolites

Figure 3 shows the sample base peak chromatogram of the grains of winter wheat variety Anthus. Compounds that were responsible for scattering in PCA results formed the interest group for the identification (Fig. 1 and Fig. 2). The characteristics of selected peaks obtained from LC-MS/MS analysis are presented in Table 1. The identification was performed by interpretation of the MS/MS fragmentation patterns of corresponding analytes, their accordance with literature data and chromatographic behavior.

Peak **1**. A molecular ion at m/z 341 had constant neutral loss (CNL) of 162 Da, which corresponds to the loss of a hexose moiety (glucose, galactose, or fructose) linked to the rest of the molecule by an O-glycosidic bond. The ions with m/z 179 and 161 indicate scission of the glycoside bond to form the complementary monohexose molecule. The other product ions, with m/z 143, 119, 131 and 101, which were present in the product ion mass spectrum from each of hexoses, are formed by CNLs of H_2O and CH_2O-group. Identified

disaccharide could be sucrose or maltose. The fragmentation behavior was consistent with the literature data [24].

Under peak no 1, lower extracted ion peak of a molecular ion with m/z 503 was found. Its MS/MS fragmentation ions at m/z 377, 323, 341 and 179 refer to the tri-hexoside, but its peak intensity was too low for further PCA. Detected tri-glycoside can be raffinose, which is found in wheat as well.

Oligosaccharides are indicators of osmotic stress resistance in plants; they tend to accumulate in a stress situation. In Figure 2, organic and winter wheat samples are clustered and separated from spring samples due to the m/z 341 and 366, m/z 341 refers to the saccharides. The content of m/z 341 in the organic and winter samples is almost twofold in comparison with the spring varieties (to the contrary of [24]). This could be due to stress conditions such as the insufficient supply of nutrients, in the case of organic samples.

Peak **2** showed a molecular ion at m/z 625. Compound 2 was tentatively identified as apigenin-6/8-C-pentoside-8/6-C-hexoside in accordance with the literature data [12,25].

Peak **3**. MS analysis showed a molecular ion at m/z 366 and a fragmentation pattern similar to those of di-C-glycosides. The MS/MS fragmentation gave CNLs of 180 (galactose or glucose), 162 (hexose), 120 (characteristic for a C-hexoside) and 42 (loss of CH_2CO group). On the basis of these results, a hexose-hexose-N-acetyl structure was proposed for compound 3.

Peaks **4** with t_r = 27.8 and 29.3 contained the same molecular ion with $[M-H]^-$ = 563 characterized by the same complex of daughter ions indicating isomers. MS/MS data showed fragments at m/z 473 and 443, indicating the presence of a C-hexosyl unit. Fragment at m/z 503 corresponds to the fragmentation of pentose. The ions at m/z 353

(aglycone+83) and 383 (aglycone+113) supported the conclusion that apigenin was the aglycone for compound 4. Therefore, its general structure could be apigenin-6-C-pentoside-8-C-hexoside, putatively shaftoside/isoschaftoside [12,25-28].

Peak **5** corresponded to a major molecular ion at m/z 468. The fragment ions included ions at m/z 332, 306, 161 and 289. We were not able to identify the compound. The similar compound (by molecular ion and fragmentation) was found in globrous canaryseed groats [29].

Peak **6** showed a molecular ion at m/z 452. The MS/MS data showed fragments at m/z 306, 316, 135, 145 and 332. A fragment ion at m/z 306 refers to the deprotonated glutathione (GSH) moiety and CNLs of 120 and 146 indicating the presence of a rhamnoside group.

Peak **7** showed an intense molecular ion at m/z 285, respective compound was putatively identified as luteolin [27].

CONCLUSIONS

Differences in the metabolomics patterns of wheat varieties could be individualized in the results of PCA. It was found that both the winter wheat grown in conventional conditions and the spring wheat grown in organic condition differ from spring wheat grown in conventional conditions of the high level of content of various oligosaccharides. This phenomenon can be explained with an exposure of osmotic stress resistance. According to our present knowledge, no such result has been reported previously in the open literature for wheat.

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Nisu (*Triticum aestivum*) sortide peakomponentide analüüs kõrgsurve-vedelikkromatograafiliste mass-spektrite alusel

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Töötati välja fikseerimata sihtmärgita metaboloomiline strateegia määramaks „nii palju väikese massiga metaboliite kui võimalik“ kolmeteistkümnes suvi-, tali- ja mahenisu (*Triticum aestivum*) sordikogumis. Metaboliidid lahutati kõrgsurve-vedelik-kromatograafiliselt pöördkolonnis (RP-HPLC), mis oli ühendatud elektronpihustus-ionisatsioon tandem-massispektromeetriga (ESI-MS/MS). Protseduur sisaldas metaboliitide ekstraktsiooni, kromatograafilist eraldamist sipelghappe ja atseetonitriili vesilahuste gradiendis, lahutatud ühendite tandem-massispektromeetrilist identifitseerimist. Erinevate nisusortide metaboloomiliste mustrite eristamine saavutati peakomponentide analüüsi meetodit (PCA) kasutades. PCA tulemused osutavad nisusortide selgele erinevusele.

Tavapärastes tingimustes kasvanud talinisu ja mahetingimustes kasvanud suvinisu erinevad tavapärastes tingimustes kasvanud suvinisust kõrgema süsivesikute sisalduse poolest. See on seletatav osmootse stressi parema taluvusega. Mahetingimustes kasvanud sordid on teistest sortidest eristatavad PCA tulemuste põhjal, mis osutab erinevate kasvatamisviiside mõjule.

Table 1. Retention times (t_r), deprotonated molecular ions and fragment ions obtained from LC/ESI-MS/MS analysis of metabolites in variety: Anthus. In the column ´Fragment ions´, base peaks are shown in bold.

Peak no	Tentative compound	$[M-H]^-$ (m/z)	t_r (min)	Fragment ions (m/z)
1	Dihexoside (unspecified)	341	1.5	**179**/161/143/119/131/101
2	Apigenin-6/8-C-pentoside-8/6-C-hexoside	625	11.9	**485**/179/221/383/323/341
3	Hexose-hexose-N-acetyl	366	23.3	**186**/204/142/246
4	Apigenin-6-C-pentoside-8-C-hexoside	563	27.8 29.3	**353**/383/443/473/503
5	Unknown	468	31.3	**332**/306/161/289
6	Rhamnoside	452	33.6	**306**/316/135/145/332
7	Luteolin	285	41.7	**241**/285/175/199/151

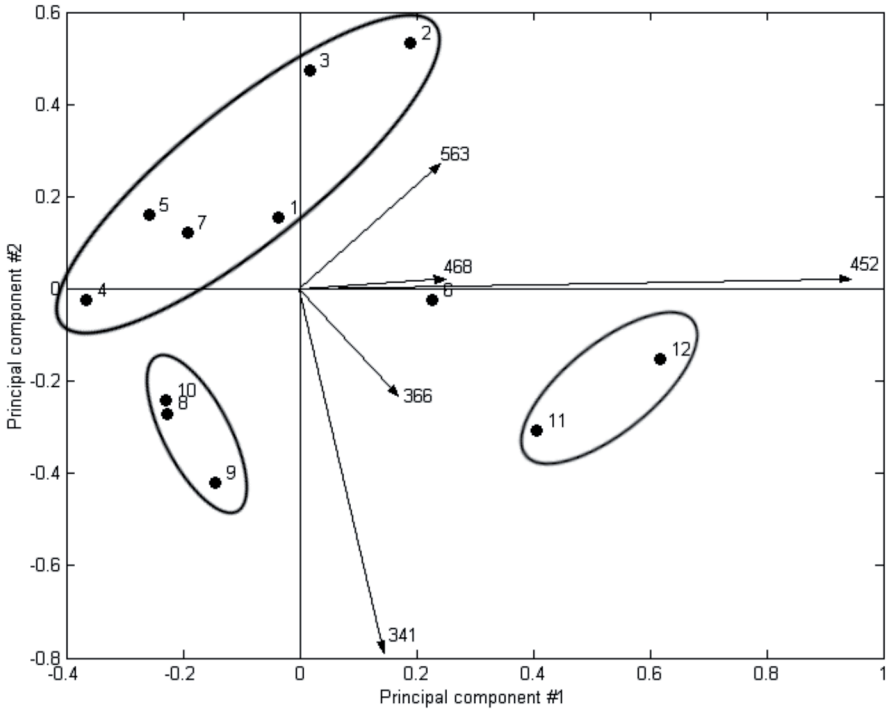

Figure 1. Representation of the LC-MS/MS base peak chromatograms of the methanol extracts of 12 wheat varieties in the first principal component coordinates (accounting for the 71% variability). Each point represents one particular chromatogram. Vectors are loadings and numbers 341, 366, 563, 468 and 452 refer to m/z values that primarily are responsible for the scattering.

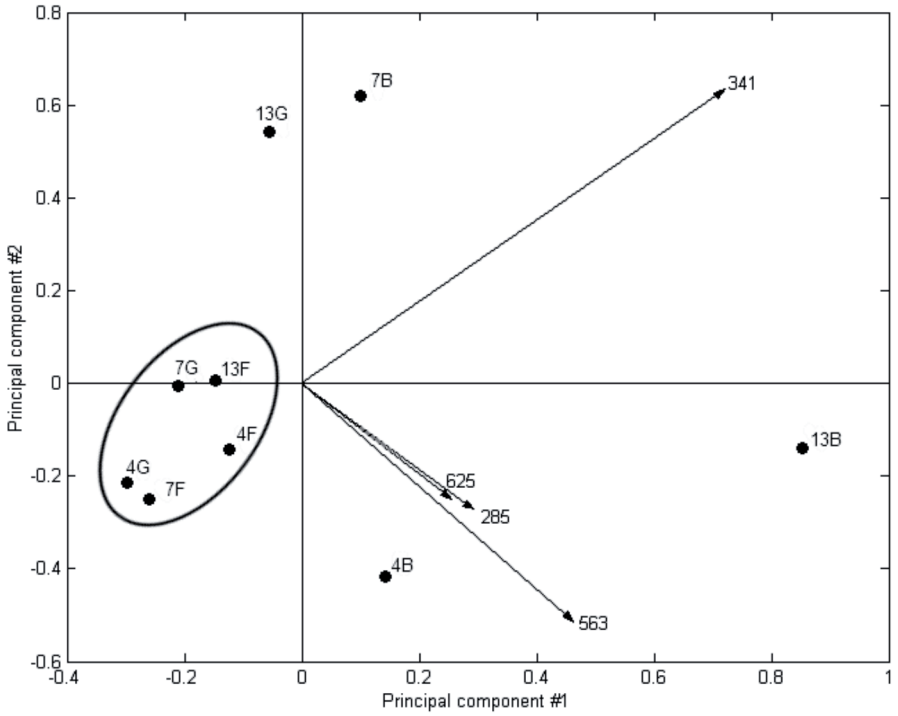

Figure 2. Representation of LC-MS/MS base peak chromatograms of the methanol extracts of grains (G), bran (B) and flour (F) of three varieties (spring-Vinjett (item 4), winter-Björke (item 7) and organic-Vinjett (item 13)) in the first principal component coordinates (accounting for the 67% variability). Each point represents one particular chromatogram. Vectors are loadings and numbers 341, 563, 285 and 625 refer to m/z values that primarily responsible for the scattering.

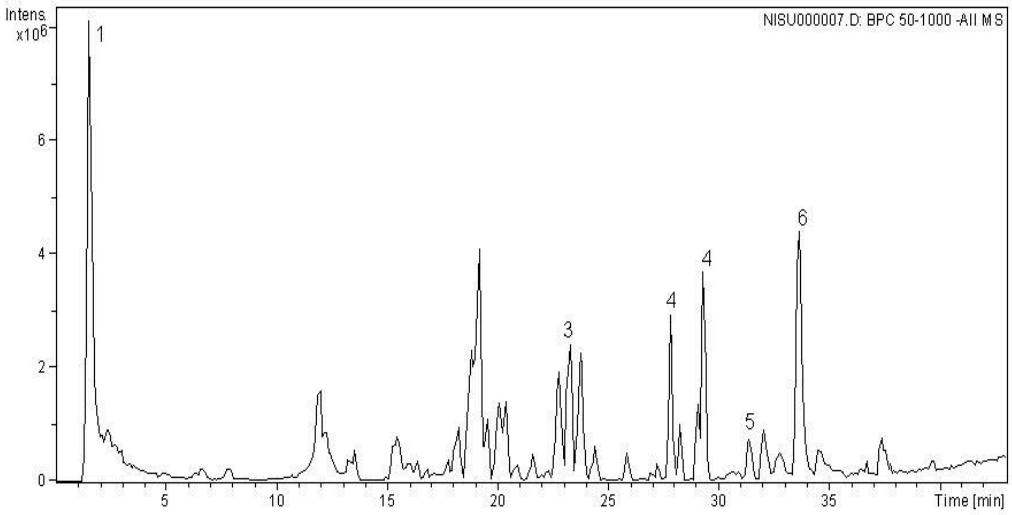

Figure 3. LC/ESI-MS/MS base peak chromatogram of the methanol extract of Anthus, a winter wheat variety. Peak numbers indicate tentative compounds, primarily responsible for the scattering in PCA. 1 – unspecified dihexoside; 3 - hexose-hexose-N-acetyl; 4 - apigenin-6-C-pentoside-8-C-hexoside; 5 – unknown; 6 - putatively rhamnoside.

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2002 - Tallinna Tehnikaülikool, keemia- ja materjalitehnoloogia õppesuund, loodusteaduste bakalaureus
1997 - Pärnu Koidula Gümnaasium, keskharidus

Täiendusõpe

2009 - European Biosafety Association, Stockholm
2008 - Avalik teenistus, Tallinn
2007 - Institute of Industrial Fermentations, Madriid
2006 - EL6-s raamprojekt SWIFT-WFD, Varssavi
2006 - EAK, Assessorite koolituskursus
2005 - EL5-s raamprojekt QUA-NAS, Barcelona
2004 - EL5-s raamprojekt QUA-NAS, Gdansk
2004 - TrainMIC, Tartu
2003 - Tartu Ülikooli Katsekoda, Tartu
2003 - Tartu Ülikooli Katsekoda, Tallinn
2003 - Quantum Eesti AS, Tartu

Teenistuskäik

2007 - …	Keskkonnaministeeriumi looduskaitse osakonna peaspetsialist
2006 - ...	Eesti Akrediteerimiskeskuse assessor
2006 - 2008	TTÜ anorgaanilise õppetooli assistent ja analüütilise õppetooli teadur
2005 - 2006	TTÜ Keemilise analüüsi laboratooriumi kvaliteedijuht
2002 - 2003	Põllumajandusuuringute Keskuse Jääkide ja saasteainete laboratooriumi juhtivspetsialist

Teadustegevus

Kapillaarelektroforees, vedelikkromatograafia, GMOd, teraviljad, peakomponentide analüüs, mass spektroskoopia

Kaitstud lõputööd

2005	magistritöö “Kapillaarelektroforeesi rakendamine südamelihase ATPaasse aktiivsuse uurimisel”
2002	bakalaureusetöö “Maatriksi mõju uurimine kroomi määramisel bioloogilises materjalis ETAAS meetodil”

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