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The mission of the JRC-IRMM is to promote a common and reliable European measurement system in support of EU policies.

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Exploratory research at IRMM 2008

Final report

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Foreword

Once again, I have the great pleasure to present to you the report on exploratory research at JRC-IRMM.

This fifth exercise was initiated with the fifth call for proposals launched in June 2007. As usual the Scientific Committee[1] of JRC-IRMM was in charge of the two-step evaluation (written applications and auditions) of the proposals, of the selection and the follow-up of those which were granted. Based on several criteria including the relevance to the institute's mission and activities, the innovative aspects, the quality of the research plan and the integration of resources, ten projects were selected, covering several of the scientific fields in which JRC-IRMM is active.

On May 13th 2009, a workshop was held where the results of seven (plus one from the previous exercise) projects were presented to the staff of the institute. Significant results have been obtained which will be of high importance both for the scientific recognition of JRC-IRMM as a research institute and for a better performance of its present and future institutional tasks. The results from six projects are shown in this report. They will obviously contribute to the development of future competencies and technical capabilities of the institute, enhancing the common goal of all our projects to create "confidence in measurements".

The outcome of this fifth year of implementation of the exploratory research system established at the IRMM is without any doubt very positive.

I wish to thank all IRMM scientists who participated in the various exploratory research projects. I would also like to acknowledge the members of our Scientific Committee for their excellent mastering of the whole process.

Alejandro Herrero
Director of IRMM

[1] Guy Bordin, Hubert Chassaigne, Philippe Corbisier, Alejandro Herrero Molina, Mikaël Hult, Sari Lehto, Josephine McCourt, Adrian Peres Bota, Arjan Plompen, Heinz Schimmel and Ursula Vincent.

COMMISSION OF THE EUROPEAN COMMUNITIES
JOINT RESEARCH CENTRE

Institute for Reference Materials and Measurements
Neutron physics

New experiments on neutron elastic scattering at GELINA

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1. SUMMARY

Interest has risen recently concerning the angular distribution of neutron elastic scattering off deuterium at low incident energies. A new R-matrix analysis resulted in angular distributions that differ in the range from 100 keV to a few MeV. These changes were detected by a worsening in the agreement between calculated and experimental results for reactor benchmarks that are used to test deuterium neutron data performance for CANDU reactors [1]. Subsequently a new theoretical approach on the basis of three-nucleon theory was made that resulted in new angular distributions that initially seemed to remedy the problem for applications. However, despite initial optimism a problem with the benchmarks remains. Experimental validation of one or the other approach in the energy range from 0.1 to 1 MeV is of interest since only few and discrepant results are found in the literature, Fig. 1, [2, 3, 4].

At the GELINA neutron time-of-flight facility [5] a new experimental setup was developed to measure elastic scattering of neutrons off deuterium in the energy range of interest. The technique proposed is complementary to the earlier works by detecting the scattered neutron instead of the recoiling deuterium. The setup is an array of three HPGe detectors, each with a ^{10}B neutron-gamma converter. This specific combination of a neutron converter and detector is novel. The performance of the system is analyzed and further use of the setup is dicussed.

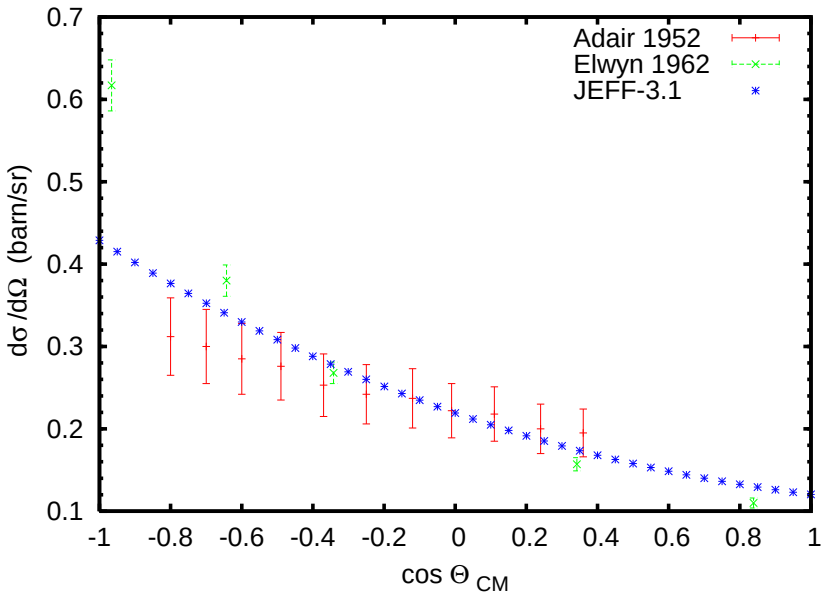

Figure 1: Measured and evaluated differential cross section for scattered neutron of 500 keV on deuterium. The larger discrepancy between evaluated and measured data is observed at backward angles.

2. INTRODUCTION

Nowadays about 50 reactors of CANDU type are spread around the world using heavy water as moderator and reflector. The low neutron capture cross section of heavy water compared to normal water makes it a good moderator for reactors using natural or low enriched uranium fuel. In such reactors it was shown that deuterium is responsible for maintaining a highly thermalized neutron flux by which the fission process may be sustained with relatively low ^{235}U enrichment.

In nuclear physics, neutron-deuteron scattering is important for testing three-nucleon forces, along with the ^{3}H and ^{3}He bound states and proton-deuteron scattering. Neutron-deuteron scattering and the triton, further offer a stage for testing models of the electro-magnetic interaction when direct Coulomb forces are absent. Theoretical studies are facilitated by the current detailed knowledge of the nucleon-nucleon (N-N) forces and the fact that the three-nucleon problem is sufficiently simple to be treated exactly.

3. EXPERIMENTAL SETUP

A new experimental setup was developed at the GELINA neutron time-of-flight facility to measure elastic scattering of neutrons on deuterium in the 100 keV-5MeV energy range. In the previous works the effort was concentrated on the detection of the recoiling deuterium. For this particular case the full reaction kinematics can be deduced from the deuterium energy measurement. In our case we decided to use another approach by detecting the emerging neutron. The choice of neutron detectors was difficult because in the energy domain of the detected neutrons most of the detectors show very low effciency, high sensitivity to gamma rays and poor stability. Taking into account the expertise of our group in measuring gamma rays produced in neutron induced reactions we decided to use germanium detectors which were fitted with $^{10}B_4C$ discs as converters. The setup consists of 3 Ge coaxial detectors placed in the horizontal plane on a table suited for angular distribution measurements. Each of the Ge detectors has a thin aluminium cap which contains a $^{10}B_4C$ disc of 80 mm diameter and 28 mm thickness. The technique benefits from the high cross section of the reaction $^{10}B(n,\alpha)^{7}Li$. In the neutron energy range of interest this reaction shows values of the cross section close to one barn. The high concentration of ^{10}B in the converter material is an advantage that is somewhat offset by the detection efficiency for the gamma-ray that is substantially less than one (about 10%).

In order to test the experimental set-up a preliminary experiment was done at the end of 2008. The neutron beam was delivered by the Gelina facility. The beam was collimated using a thick copper cylinder of 200 mm with an inner diameter of 70 mm. The angular distribution table was placed 300 m from the neutron producing target. The Ge detectors were placed at 45, 90, and 130 degrees with respect to the beam line and at an average distance from the target of about 250 mm. The sample was graphite with a thickness of 10.2 mm and a diameter of 80 mm. The obtained gamma spectrum of one of the germanium detector is presented in figure 2.

The Doppler broadening of the 478 keV line is due to the ^{7}Li recoil in the converter. Despite this effect, it can be clearly seen that the gamma line of interest can be separated from the adjacent gamma lines.

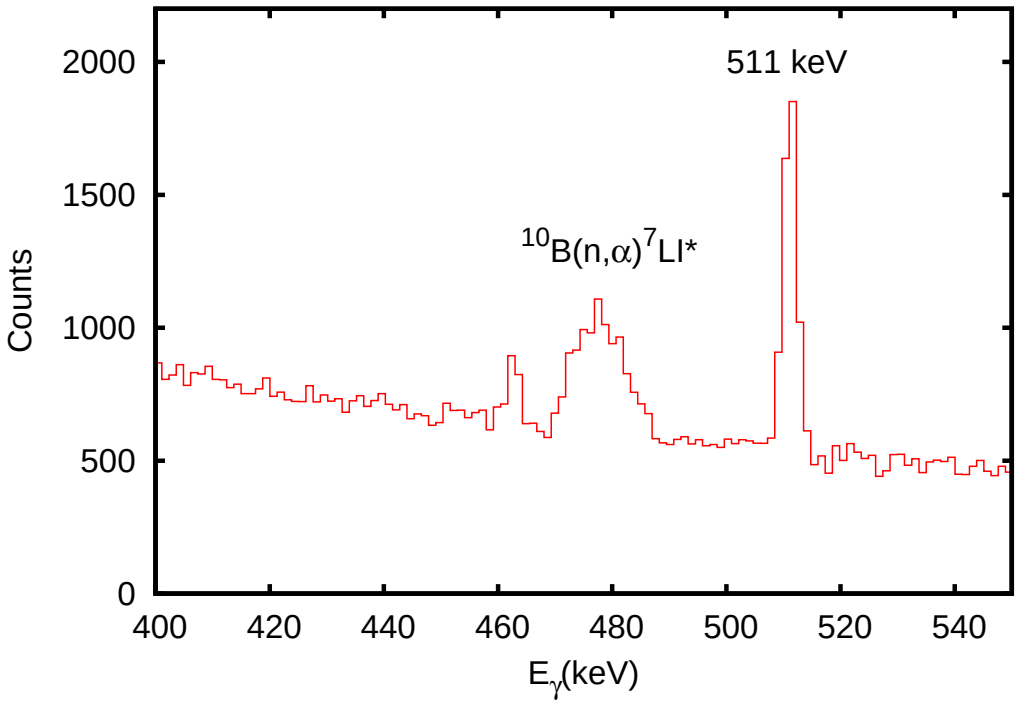

Figure 2: Gamma spectrum showing the line produced in the $^{10}B(n,\alpha)^{7}Li^*$ reaction. The broadening of the 478 Kev gamma ray can be observed.

It was verified that without converter disks there is no structure in the pulse height spectrum at the location of the 478 keV line. However, with the converters in place and the sample removed we did observe a signal that resulted, most likely, from inadequate collimation. This background can be subtracted but its determination increases the measurement time and reduces the accuracy. What we can test is the differential cross section of neutron elastic scattering on carbon. The results are presented in figure.3 which shows the ratio between the number of neutrons detected at 90 and 130 degrees as a function of neutron energy. The data are compared with the ENDF/B-VII data base for carbon. The preliminary experimental data are in good agreement with the evaluated data. The error bars are mainly due to the bad collimation of the beam and are estimated roughly to 30%. After the test a new collimation system has been mounted, which will reduce the error bars that are due to the beam spread to a desired value of 5%.

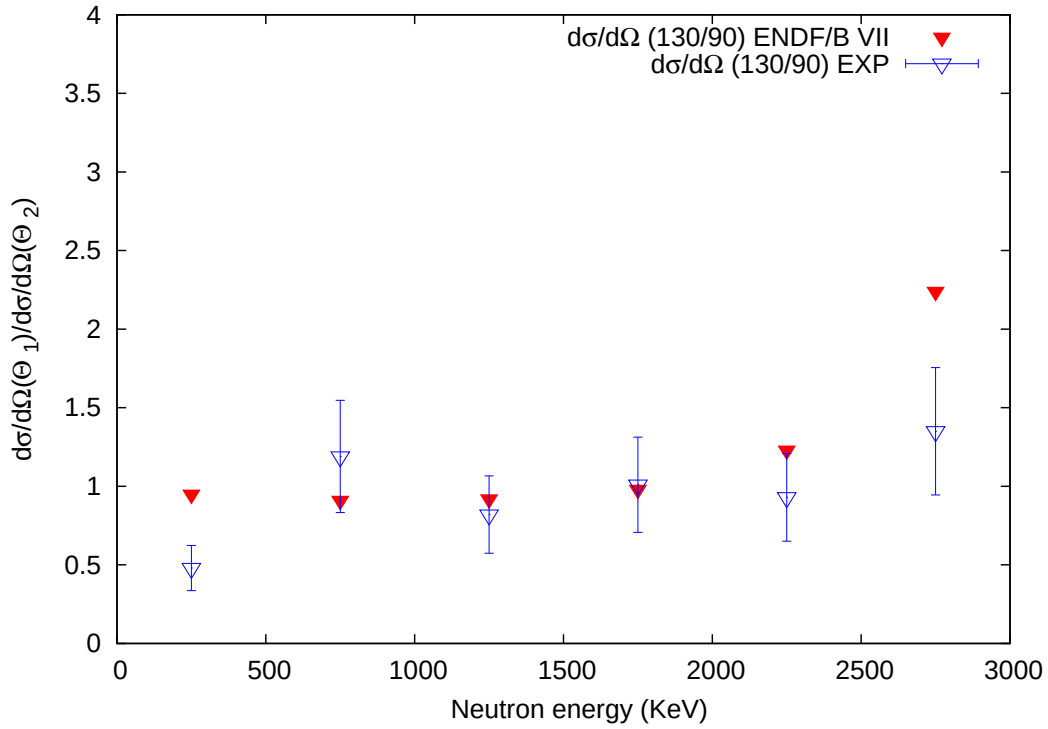

Figure 3: Ratio between diferential cross section of elastic scattering of neutron on carbon target

4. CONCLUSIONS

A new setup was built to measure the elastic scattering of neutrons on deuteron and was preliminarily tested with a graphite target. The obtained data seems to be in reasonably good agreement with the evaluated data. Setup improvements have been implemented and are currently being tested by new measurements on graphite. In the near future a C6D6 target will be mounted and the diferential cross section on deuterium will be measured aiming for an accuracy of better than 8 %.

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Institute for Reference Materials and Measurements
Food safety and quality

Coupling of High Performance Liquid Chromatography to Mass Selective Detection by Atmospheric Pressure Laser Ionisation to Determine 15+1 EU Priority PAHs in Food

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1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are the largest group of chemical compounds with known carcinogenic properties. Exposure to PAHs happens mainly via ambient air, drinking water, and food. For a non-smoking human, food has been identified as the main source of exposure to PAHs [1].

Some decades ago the United States Environmental Protection Agency published an analytical method for the determination of the "16 most frequently found PAHs", known as the 16 EPA PAHs, which lead to focus research on these 16 substances [2]. In 2002 the European Commission's Scientific Committee on Food (SCF) assessed toxicological data on 33 PAHs and concluded that 15 of the PAHs covered were of concern to human health [1]. This group of 15 SCF-PAHs included 8 of the 16 EPA PAHs. In 2005 the European Commission recommended monitoring of the 15 SCF PAHs in various food matrices [3]. Also in 2005, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) requested including benzo[*c*]fluorene in occurrence monitoring programs [4]. These events led to a call for data on the combined set of 15+1 EU priority PAHs (Table 1) by the European Food Safety Authority (EFSA) [5, 6].

Up to this time PAHs had been quantified mainly by gas chromatography with mass selective detection (GC-MSD) and high performance liquid chromatography with fluorescence detection (HPLC-FLD) [7]. The change from the 16 EPA PAHs to the 15+1 EU priority PAHs implied adaptation of existing analytical procedures. Challenges in case of GC-MS analysis include overlapping of analyte peaks and low volatility of the high molecular mass PAHs causing mass transfer problems. HPLC-FLD analysis suffers from the low fluorescence of some target compounds respectively absence of fluorescence in case of cyclopenta[*cd*]pyrene. The analytical community reacted, modified methods and published results on the determination of the EU priority PAHs in various matrices [8-15]. However most of the cited papers address GC-MS methods.

So far not all issues concerning the resolution of homologous PAHs and/or non-satisfactory limits of detection (LODs) could be resolved. It was practically impossible to separate the non-target PAH triphenylene from chrysene on GC-columns, which enabled the separation of the 15+1 EU priority PAHs. Also the sensitivity issue with HPLC-FLD measurements could not be solved yet. They still give unacceptably high LODs for the two analytes cyclopenta[*cd*]pyrene and benzo[*j*]fluoranthene.

Liquid chromatography with mass spectrometric detection (LC-MS) recently found recently its way into food control laboratories. This technique is going to supersede classical detection systems such as UV, or fluorescence spectroscopy in many fields of application. Reasons are broader applicability, since it does not rely on chromophores, and high selectivity. In theory, LC-MS could combine the advantages of both HPLC and mass spectroscopy with regard to mass transfer after injection, and sensitivity and selectivity of detection.

However the most serious limitations of LC-MS are caused in the interface that connects the high performance liquid chromatograph with the mass spectrometer. The objective of this device is to ionise and transfer the analytes as selectively as possible from a few hundreds of microliter per minute of an aqueous/organic effluent of the HPLC into the high vacuum of the MS. The ionisation process is most critical in that respect, which is unfortunately prone to interferences coming from the matrix. Hence technical development has been focusing since the early days of LC-MS on the design of suitable interfaces. Nowadays electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) interfaces are mainly applied in LC-MS. Electrospray ionisation is based on the protonation respectively attachment of cations such as sodium to the molecule of interest. This technique works well for polar analytes. Apolar substances such as polycyclic aromatic hydrocarbons cannot be ionised through this mechanism. The second most frequently applied ionisation mechanism, APCI, is based on the full

vaporisation of the HPLC effluent and gas phase ionisation of the analytes by means of electrons emitted from a corona discharge needle. This ionisation technique is more appropriate for PAHs. However, also APCI does not provide sufficient sensitivity for the 15+1 EU priority PAHs for applying it for routine analysis of food.

Atmospheric pressure photo ionisation (APPI) is an alternative ionisation technique, which is frequently applied for the ionisation of photoactive substances such as PAHs. The ionisation is based on the gas phase interaction of the target molecule with photons emitted from a vacuum UV (VUV) source. Such single photon ionisation (SPI) results in the formation of a positively charged molecule ion and the emission of an electron (see Equation 1) [16]. The boundary condition for ionisation is that the ionisation energy of the target molecule must be below the energy of the photon. However, the ionisation efficiency may be increased for many photoactive substances by the use of an excess of a readily ionisable dopant that acts as an intermediate in the ionisation reaction [17]. The dopant "conserves" the energy of the photons that is otherwise easily lost by collision with gas-phase constituents of the atmospheric pressure interface, and transfers it via solvent molecules to the analyte. For chromatographic reasons the dopant is added post-column. Analyte ionisation may occur via either proton transfer or charge exchange reactions. The former is described in Equations 2 to 6 [18]. It consist first of the photoionisation of the dopant, which is immediately surrounded by a cluster of polar solvent molecules (e.g. methanol, or acetonitrile). In a second step the charge of the dopant is transferred via a proton to the solvent cluster and finally via another proton transfer reaction to the analyte. To allow charge exchange reactions by electron transfer the ionisation potential (IP) of the analyte must be lower than that of the dopant. The IPs of PAHs are roughly in the range of 7.1 eV to 8.2 eV. Suitable dopants must have IPs above that range. Additionally their radical cations must be stable in the presence of mobile phase constituents such as methanol or acetonitrile. The ionisation process by charge exchange is presented in Equation 7 and 8. The two ionisation processes can be distinguished in the mass spectrum, because the former would give quasi-molecular ions with the mass M+1, while charge exchange reactions will lead to molecular ions with mass M. However it should be noted that the ionisation process described in Equations 1 to 8 is simplified and that other ionisation reactions respectively quenching reactions might take place depending on the properties of the target analytes, the matrix and of the mobile phase of the HPLC [19].

without dopant

$$A + h\nu \rightarrow A^+ + e^- \quad (1)$$

with dopant

Proton transfer

$$D + h\nu \rightarrow D^+ + e^- \quad (2)$$

$$D^+ + S \rightarrow D^+(S) \quad (3)$$

$$D^+(S)_{n-1} + S \leftrightarrow D^+(S)_n \quad (4)$$

$$D^+(S)_n \leftrightarrow D + SH^+(S)_{n-1} + \text{(D-H)} \quad (5)$$

$$SH^+(S)_{n-1} + A \leftrightarrow AH^+(S)_m + (1 + n - m)S \quad (6)$$

Charge transfer

$$D + h\nu \rightarrow D^+ + e^- \quad (7)$$

$$D^+ + A \rightarrow A^+ + D \quad (8)$$

where A stands for the analyte molecule, hν denotes the energy of the photon, AH^+ the protonated molecular ion of the analyte and e^- an electron, D the dopant molecule, D^+ the positively charged dopant molecule, S the solvent, $(S)_n$ a cluster of n solvent molecules, SH^+ the protonated solvent molecule

Another technique based on ionisation with photons is resonance enhanced multi-photon ionisation (REMPI). REMPI has been applied since almost ten years in combination with GC-MS for the determination of PAHs in waste incineration gases, urban dust, and tobacco [20-22]. This technique uses UV light emitted from a laser source for the ionisation of the target molecules. In contrast to photo ionisation REMPI is based on an m+n photon reaction. The ionisation process is described in Equation 9 and 10.

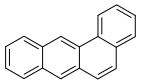
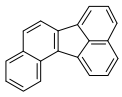
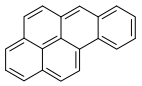
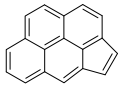
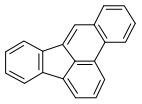
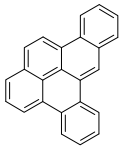
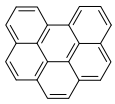
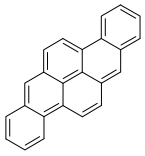
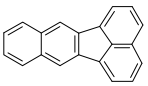
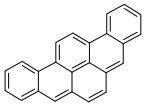
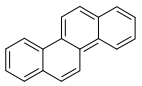
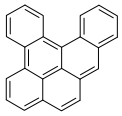
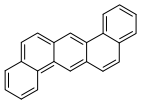
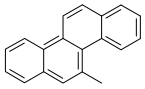
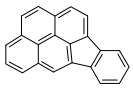
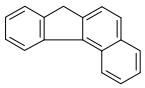
$$A + mh\nu \rightarrow A^* \tag{9}$$

$$A^* + nh\nu \rightarrow A^+ + e^- \tag{10}$$

More recently atmospheric pressure laser ionisation (APLI) has been used for the hyphenation of high performance liquid chromatography (HPLC) with MSD [23, 24]. Constapel et al. described the ionisation of polyaromatic compounds by 1+1 photon APLI. They state that the photon flux within the ionisation volume is in APLI (in contrary to APPI) independent of the mobile phase composition [23]. Compared to APCI-MS they found in APLI-MS an increase of the signal abundance by at least two orders of magnitude for the same amounts of substance infused into the respected interfaces. The respective APLI-MS system consisted of a fixed wavelength (248 nm) KrF excimer laser operated at 100 Hz repetition rate, 15 mJ pulse energy and 8 ns pulse duration. The laser was connected to a custom-made APLI source that was connected to a Micromass Q-TOF mass spectrometer. However, only solutions of the analytes in pure solvent were introduced into the APLI-interface via either syringe pumps or the sample loop of an HPLC system. Lorenz et al. explained the outstanding detection capability of their APLI system compared to APPI by the homogeneous photon flux through the ionisation volume, whereas photons of the VUV source of the APPI interface are consumed by the solvent/matrix already within a few millimetres from the lamp surface [24]. Unfortunately, the experiments reported in both publications were limited to model substances in solvent solutions. Information on the potential influence of matrix constituents of e.g. food on the ionisation process was not at all provided.

The objective of this exploratory research project was to investigate the potential of HPLC-APLI-MS to identify and quantify the 15+1 EU priority PAHs in food products. The results were compared to HPLC-dopant assisted (DA)-APPI-MS and HPC-FLD measurements. The method performance parameters limit of detection and limit of quantification were determined in solvent solution. The influence of matrix constituents on the ionisation process was assessed using spiked matrix extracts. The names, acronyms and chemical structures of the target analytes are given in Table 1.

Table 1: 15 + 1 EU priority PAHs under investigation (with acronyms)

1	Benzo[*a*] anthracene (BaA)		9	Benzo[*j*] fluoranthene (BjF)	
2	Benzo[*a*] pyrene (BaP)		10	Cyclopenta [*cd*]pyrene (CPP)	
3	Benzo[*b*] fluoranthene (BbF)		11	Dibenzo [*a,e*]pyrene (DeP)	
4	Benzo[*ghi*] perylene (BgP)		12	Dibenzo [*a,h*]pyrene (DhP)	
5	Benzo[*k*] fluoranthene (BkF)		13	Dibenzo [*a,i*]pyrene (DiP)	
6	Chrysene (CHR)		14	Dibenzo [*a,l*]pyrene (DlP)	
7	Dibenzo[*a,h*] anthracene (DhA)		15	5-Methyl-chrysene (5MC)	
8	Indeno[*1,2,3-cd*]pyrene (IcP)		+ 1	Benzo[*c*] fluorene (BcL)	

2. EXPERIMENTAL SETUP

The experiments with HPLC-DA-APPI-MS and HPLC-FLD were carried out at IRMM with own instrumentation. The acquisition of an APLI interface compatible with existing LC-MS instruments was planned, but was in the course of the purchase process abandoned due to several reasons, such as proprietary rights and a significant increase of the costs of the interface. However as an alternative to the purchase of the APLI interface it was agreed with the patent holder (Bruker Daltonics) to run a set of experiments on their demo-instrument for evaluating whether or not the particular APLI interface would significantly increase the detectability of PAHs comapared to APPI-MS and FLD.

3. MATERIALS AND METHODS

3.1. Chemicals

Acetonitrile (ACN), cyclohexane, *n*-hexane, anisole, and 2-propanol (iPrOH) were all of analytical grade or higher (VWR, Darmstadt, Germany).

The analytes benz[*a*]anthracene (BaA) CAS# 56-55-3, chrysene (CHR) CAS#218-01-9, 5-methylchrysene (5MC) CAS# 3697-24-3, benzo[*b*]fluoranthene (BbF) CAS#205-99-2, benzo[*j*]fluoranthene (BjF) CAS#205-82-3, benzo[*k*]fluoranthene (BkF) CAS#207-08-9, benzo[*a*]pyrene (BaP) CAS#50-32-8, indeno[1,2,3-*cd*]pyrene (IcP) CAS#193-39-5, dibenz[*a,h*]anthracene (DhA) CAS#53-70-3, benzo[*ghi*]perylene (BgP) CAS#191-24-2, dibenzo[*a,*l]pyrene (DlP) CAS#191-30-0, dibenzo[*a,e*]pyrene (DeP) CAS#192-65-4, and dibenzo[*a,h*]pyrene (DhP) CAS#189-64-0, were BCR reference materials and obtained from the Institute for Reference Materials and Measurements (IRMM) of the European Commission's Joint Research Centre (Geel, Belgium).

Cyclopenta[*cd*]pyrene (CPP) CAS#27208-37-3, purity >99.0 % by GC, was manufactured on request (Biochemisches Institut für Umweltkarzinogene, Großhansdorf, Germany). Neat benzo[c]fluorene (CAS#205-12-9, purity 98.2 %) was supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany) and dibenzo[*a,i*]pyrene (DiP) CAS#189-55-9 were supplied by Campro Scientific GmbH (Berlin, Germany).

Isotopically labelled internal standards $^{2}H_3$-5-methylchrysene and $^{13}C_{12}$-dibenzo[*a,i*]pyrene were purchased from Cambridge Isotope Labs, Andover, MA, USA. The carbon-13 labelled substances $^{13}C_6$-benz[*a*]anthracene, $^{13}C_4$-benzo[*a*]pyrene, $^{13}C_6$-benzo[*b*]fluoranthene, $^{13}C_6$-benzo[*k*]fluoranthene, $^{13}C_{12}$-benzo[*ghi*]perylene, $^{13}C_6$-chrysene, $^{13}C_6$-dibenz[*a,h*]anthracene, $^{13}C_6$ dibenzo[*a,e*]pyrene, and $^{13}C_6$-indeno[1,2,3-*cd*]pyrene were obtained from Cerilliant Cooperation (Round Rock, Texas, USA) and 9-fluoro-benzo[*k*]fluoranthene was purchased from Chiron AS (Trondheim, Norway).

The mixed standard solution PAH-MIX 183, containing all 15+1 EU priority PAHs at a level of 10 µg/mL was supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany).

3.2. Preparation of standards for instrument calibration

A stock standard solution containing the 15+1 EU priority PAHs at a level of 200 ng/mL was prepared gravimetrically by dilution of PAH-MIX 183 (Dr. Ehrenstorfer) in iso-propanol (iPrOH). This stock standard solution was used for the preparation of standard solutions for instrument calibration in both acetonitrile/water (80/20 v/v) and olive pomace oil extract. The purpose of the latter was to investigate matrix effects.

Isotopic labelled and fluorinated PAH standards (Table 2) were used as internal standards in mass spectrometric measurements.

3.2.1. Calibration standards in solvent

Standards for instrument calibration were prepared in acetonitrile. The concentration of the standards in solvent solution was 0.4 ng/ml, 1.1 ng/ml, 2.9 ng/ml, 7.1 ng/ml, 21.4 ng/ml, and 35.7 ng/ml. Each standard was injected in triplicate.

Table 2: Isotopically substituted and fluorinated internal standards

no	IUPAC name	molecular mass (g/mol)	acronym
1	$^{2}H_3$-5-methylchrysene	245	H-5MC
2	$^{13}C_6$-benz[*a*]anthracene	234	C-BaA
3	$^{13}C_4$-benzo[*a*]pyrene	256	C-BaP
4	$^{13}C_6$-benzo[*b*]fluoranthene	258	C-BbF
6	$^{13}C_6$-benzo[*k*]fluoranthene	258	C-BkF
7	$^{13}C_{12}$-benzo[*ghi*]perylene	288	C-BgP
8	$^{13}C_6$-chrysene	234	C-CHR
10	$^{13}C_6$-dibenz[*a,h*]anthracene	284	C-DhA
11	$^{13}C_6$-dibenzo[*a,e*]pyrene	308	C-DeP
13	$^{13}C_{12}$-dibenzo[*a,i*]pyrene	314	C-DiP
15	$^{13}C_6$-indeno[1,2,3-*cd*]pyrene	282	C-IcP
X	9-fluoro-benzo[*k*]fluoranthene	270	F-BkF

3.2.2. Matrix matched calibration standards

Blank olive pomace oil extracts were prepared from commercial olive pomace oil, which was checked prior to use for the absence of PAHs, by donor-acceptor-complex-chromatography (DACC). The set up of the DACC method is described in detail in ISO 22595:2009. A brief description of the sample preparation by DACC is given below. The DACC extraction of the olive pomace oil was repeated 100 times; the extracts were pooled, and in the following evaporated at 40ºC under a gentle stream of nitrogen to dryness. The extract was then reconstituted in 10 mL of ACN/H2O (ratio = 8/2), and used for the preparation of the matrix matched calibration standards.

The levels of the matrix matched standards were 0.0 ng/mL, 0.4 ng/mL, 1.1 ng/mL, 4.3 ng/mL, 12.7 ng/mL, and 44.1 ng/mL of the individual EU priority PAHs.

3.2.3. Calibration standards for HPLC-FLD measurements

A portion was taken from all standard preparation for analysis by HPLC-FLD. The rest of the standard preparations were spiked with the isotopic labelled standards before splitting into two portions, one of each was intended for analysis by HPLC-DA-APPI-MS, the other by HPLC-APLI-MS.

3.3. Preparation of the sample of FAPAS® round 0630 and BCR-458

The sample of the proficiency test FAPAS® round 0630 (16 EU PAHs in olive pomace oil) consists of a very complex matrix. It serves for this reason in our laboratory as a reference sample to evaluate the efficiency of sample clean up processes. In this project it was used to evaluate the efficiency and selectivity of the analyte ionisation in the respective mass spectrometer interfaces. BCR-458 consists of spiked coconut oil certified for the contents of 5 target analytes (BaP, BkF,

BgP, CHR, and ICP). This is the only certified reference material for PAHs in edible oil on the market. However the matrix of this sample is less complex than olive pomace oil.

The sample preparation for both samples was carried out by donor-acceptor complex chromatography (DACC) as described before. Ten replicate samples were prepared, the extracts pooled, evaporated and reconstituted in 1 mL of ACN/H_2O (ratio = 8/2). The exctract was split into two portions and analysed by both LC-DA-APPI-MA and LC-APLI-MS.

3.4. Instruments and methods

3.4.1. Donor-acceptor-complex-chromatography

An Agilent 1100 system equipped with an isocratic and a quaternary pump, two splitting valves, diode array, and fluorescence detector was used for the sample preparation by DACC (Agilent Technologies, Santa Clara, CA, USA)). The DACC column was a Chromsphere PI, 80 mm length and 3 mm internal diameter, 5 µm particle size, (Chrompack, Bergen Op Zoom, NL). The PAHs containing fraction was isolated with an Agilent 1200 fraction collector (Agilent N.V.).

The DACC method is described in ISO standard 22595:2009.

In short, the olive pomace oil was diluted with iPrOH to a ratio of 7/3. A total volume of 1 ml was injected onto the DACC column in 5 consecutive injections of 200 µl, each in time intervals of 3 minutes. The loading and elution of the fat matrix was carried out in forward direction at an iPrOH flow rate of 1 ml/min. The elution of the oil background was monitored by diode-array-detection (DAD) at 242 nm and 254 nm. After 40 min of run time the DACC column was switched into back-flush mode in order to elute within a narrow band the fraction in which the target analytes were expected. Elution of this fraction was supported by a quaternary HPLC pump and carried out at a flow rate of 2.5 ml/min with a mixture of ethyl acetate (EtOAc) and ACN (ratio = 7/3). The eluate between the run time 40.10 and 41.75 minutes was collected.

3.4.2. APPI measurments

LC-DA-APPI-MS experiments were carried out using an Accela high speed LC system coupled to a Finnigan TSQ Quantum Ultra mass spectrometer (both Thermo Fisher Scientific, Zellik, Belgium). A Finnigan Ion Max APCI/APPI combination probe was applied as interface between the LC and the MS. A Shimadzu LC-20AD pump (Shimadzu Benelux N.V., Deurne, Belgium) was integrated in the system for post-column dopant supply.

An LC flow rate of 700 µL/min was found most appropriate with regard to analysis time and ionisation efficiency. A gradient of the mobile phase was applied for chromatographic separation of the analytes. The initial eluent consisted of methanol with 8 % water (kept until 1 min), then the methanol content of the mobile phase was linearly increased to 100 % within 8 minutes. In the following ethyl acetate was added to the methanol in a linear gradient to reach 65% ethyl acetate in 7 min followed by an isocratic period (2 min) with the same eluent composition. This was necessary to elute the dibenzopyrenes. After this, the gradient was directed back to initial composition and the column was flushed and equilibrated for 5 minutes before the next injection. The injection volume was 25 µl and the column temperature was 30ºC

The parameter setting for the gas flows in the mass spectrometer were: sheath gas pressure 20 mtorr, auxiliary gas pressure 5 mtorr, and collision gas pressure off. An ion sweep gas flow was not applied. The tube lens values varied between 65 V and 120 V depending on the respective substance. The skimmer offset value was 0 V. The temperatures settings of the APPI/APCI probe were 400°C vaporiser and 350°C capillary temperature. Anisole was used as dopant at 30 µl/min flow rate. The analyte signals were recoreded in 5 time windows in order to increase the number

of measurement cycles and hence the number of data points per peak. The switching times were 3.75 min, 6.4 min, 11.8 min, and 15.0 min respectively.

3.4.3. APLI measurments

The LC-APLI-MS measurements were performed in the facilities of Bruker Daltonics in Bremen (Germany), using a Bruker micrOTOF time of flight mass spectrometer that was equipped with a Bruker APLI source. Chromatography was performed with an Agilent 1200 series binary HPLC pump (Agilent Technologies) and a Pinnacle II PAH column of dimension 250 mm x 2.1 mm, 5 µm particle size (Restek, Bad Homburg, Germany). Gradient elution was performed at a flow rate of 0.35 mL/min starting with a binary solvent mixture of 80 % ACN and 20 % water, and increasing the ACN content to 85 % within 30 min, followed by a steeper gradient to 100 % ACN in the next 10 min. The total runtime was 60 min and the column temperature was kept during the whole run at 40 °C. The injection volume was 20 µL. The APLI interface consisted of a KrF laser (wave length 248 nm), which was operated at 100 Hz with a puls energy of 15 mJ/pulse. The mass spectrometer was programmed to record mass-to-charge (m/z) ratios from 50 to 500 at a resolution of 0.1 m/z.

3.4.4. HPLC-FLD measurements

HPLC-FLD experiments were carried out using an Agilent 1200 rapid resolution LC system. Separation method and analytical column (Varian Chromsphere PAH, 250mm x 2 mm, 5 µm particle size) were as applied in HPLC-DA-APPI-MS experiments, despite that the organic modifier of the mobile phase was acetonitrile instead of methanol. The latter gives better sensitivity in HPLC-DA-APPI-MS. However a better baseline, better peak shapes, and reduced column load with regard to pressure can be achieved when acetonitrile is applied as eluent. Hence ACN was chosen for HPLC measurements. The ratio of acetonitrile in the mobile phase was adjusted according to the solvent elution strength normogram in order to achieve the same elution strength value for the acetonitrile/water mixture just as for the methanol/water mixture. The same applies also for the acetonitrile and methanol/ethylacetate mixture. As a consequence of the equal elution strength values of the initial mobile phases, the same gradient parameters e.g. gradient volume, gradient slope were set for the HPLC-FLD experiments as was applied for the HPLC-DA-APPI-MS measurements. An LC flow rate of 700 µL/min was set and the following gradient program was applied: the initial eluent consisted of acetonitrile with 15 % water (kept until 1 min), then the acetonitrile content of the mobile phase was linearly increased to 100 % within 8 minutes. In the following ethylacetate was added to the acetonitrile in a linear gradient to reach 70% ethyl acetate in 7 min followed by an isocratic period (2 min) with the same eluent composition. After this, the gradient was directed back to initial composition and the column was flushed and equilibrated for 5 minutes before the next injection. The injection volume was 10 µl and the column temperature was 30 ºC.

Detection was carried out by Agilent 1200 fluorescence detector at a fixed excitation wavelength of 270 nm and four emission wavelenghts were recorded corresponding to the fluorescence properties of the target analytes. These were 380nm (for BcL, CHR and 5MC), 420 nm (for BaA, BkF, BaP, DlP, DhA, BgP and DeP), 450 nm (for BbF, diP and DhP) and 508 nm (for BjF and IcP) respectively. The photomultiplier gain value was set to 10.

4. RESULTS AND DISCUSSION

4.1. Calibration standards in solvent

Each instrument was calibrated at six different concentration levels with standards in acetonitrile stemming from the same preparation. Each standard was injected in triplicate.

Evaluation criteria were linearity and the instrument detection limit (IDL).

Linearity of the calibration curves was proven for each analysis technique over the whole calibration range by performing Mandel's tests. The coefficient of determination (R^2) was for all analytes and techniques in the range of 0.99.

The IDLs were calculated according to ISO Standard 11843-2:2000. However it has to be noticed that the magnitude of the given IDL is influenced by many instrumental factors that determine the quality of the analyses. Hence the calculated IDLs might be meaningful only for the particular instrument at the time when the experiments were performed and are likely to change with a different instrument. Therefore the discussion of the results will focus on the order of magnitude of the IDLs rather than at precise figures. Table 3 contains the IDLs of the 15+1 EU priority PAHs for the three investigated analysis techniques. The given concentration values are not normalised for the injection volumes, which varied between 10 µL (HPLC-FLD) and 25 µL (HPLC-DA-APPI-MS). However, this is considered in the second column, which lists for each analyte-analysis technique combination the amount of substance on column.

The difference in performance is marginal for the currently regulated PAH, benzo[*a*]pyrene (BaP). The lowest and highest IDLs for BaP differ by less than one pico-gram. However there is not any general rule that would descibe the performance of one or the other analysis method. For many analytes fluorescence detection gave lowest IDLs. This is in particular the case for traditional PAHs, which means PAHs that are also on the priority list of the US Environmental Protection Agency (US EPA). For other compounds such as BcL, BjF, CPP, or the dibenzopyrenes the situation is different. Benzo[*j*]fluoranthene shows only weak fluorescence. Therefore the IDL of the HPLC-FLD system is significantly higher than the respective values of the other two measurement systems. Cyclopenta[*cd*]pyrene can neither be determined by HPLC-FLD nor by LC-APLI-MS. This indicates that the excitation respectively laser ionisation processes have from a mechanistic point of view some commonalities. With both techniques the analyte molecules take up energy from light and get into an excited state. However in fluorescence the excited molecule returns to its ground state by emitting a photon, whereas it gets ionised by taking up another photon in APLI. For CPP the intermediate, excited state does not seem to be stable enough to fluoresce respectively to take up the second photon in APLI. Intra- respectively intermolecular relaxation processes seem to be dominant. However looking to BjF, it becomes obvious that the mechanisms are more diverse than this explanation would suggest. The IDL of HPLC-FLD is for BjF one order of magnitude larger than that of HPLC-APLI-MS. This indicates, as mentioned before, weak fluorescence, but good ionisation efficiency of BjF. Hence the lifetime of the excited state of BjF is apparently sufficiently long to take up a second photon, which let us conclude that fluorescence is superseded by radiation-free losses of energy. The high IDLs of LC-APLI-MS for the dibenzopyrenes is reasoned by on the one hand absence of analyte signals at the lowest concentration level and on the other hand by lower precision of the replicate injections, which could have been caused by chromatographic problems.

The recorded analyte signals corresponded in HPLC-DA-APPI-MS to the mass of the molecular ion, which indicates a charge transfer ionisation mechanisme. The achieved IDLs are for most analytes in the range of one to two times of the IDLs of HPLC-APLI-MS. A significant difference was found for the dibenzopyrenes, which, as said before, might have been influenced by sub-optimal chromatography. However the application of HPLC-DA-APPI-MS enables the liquid chromatographic mass spectrometric determination of CPP. Hence this is to our best

knowledge the only liquid chromatographic analysis technique that allows the determination of the whole set of 15+1 EU priority PAHs using only one detection sysem.

Table 3: Instrument detection limits (IDL) according ISO 11843-2:2000 for the 15+1 EU priority PAHs in solvent solution

Analyte	HPLC-FLD		HPLC-APLI-MS		HPLC-DA-APPI-MS	
	ng/mL	pg on column	ng/mL	pg on column	ng/mL	pg on column
BcL	0.88	8.8	0.27	5.4	12.90	322.5
BaA	0.16	1.6	0.31	6.2	0.69	17.3
CHR	0.27	2.7	0.26	5.2	0.30	7.5
5MC	0.25	2.5	0.27	5.4	0.17	4.3
BaP	0.39	3.9	0.22	4.4	0.19	4.8
BbF	0.28	2.8	0.13	2.6	0.80	20.0
BjF	3.42	34.2	0.16	3.2	0.41	10.3
BkF	0.23	2.3	0.22	4.4	0.36	9.0
BgP	0.67	6.7	0.14	2.8	0.49	12.3
IcP	1.76	17.6	1.26	25.2	1.24	31.0
DhA	0.33	3.3	0.38	7.6	0.24	6.0
DeP	0.24	2.4	1.04	20.8	0.33	8.3
DhP	1.44	14.4	2.44	48.8	0.11	2.8
DiP	0.55	5.5	2.15	43.0	0.07	1.8
DlP	0.44	4.4	2.25	45.0	0.14	3.5
CPP	not detected		not detected		1.52	38.0

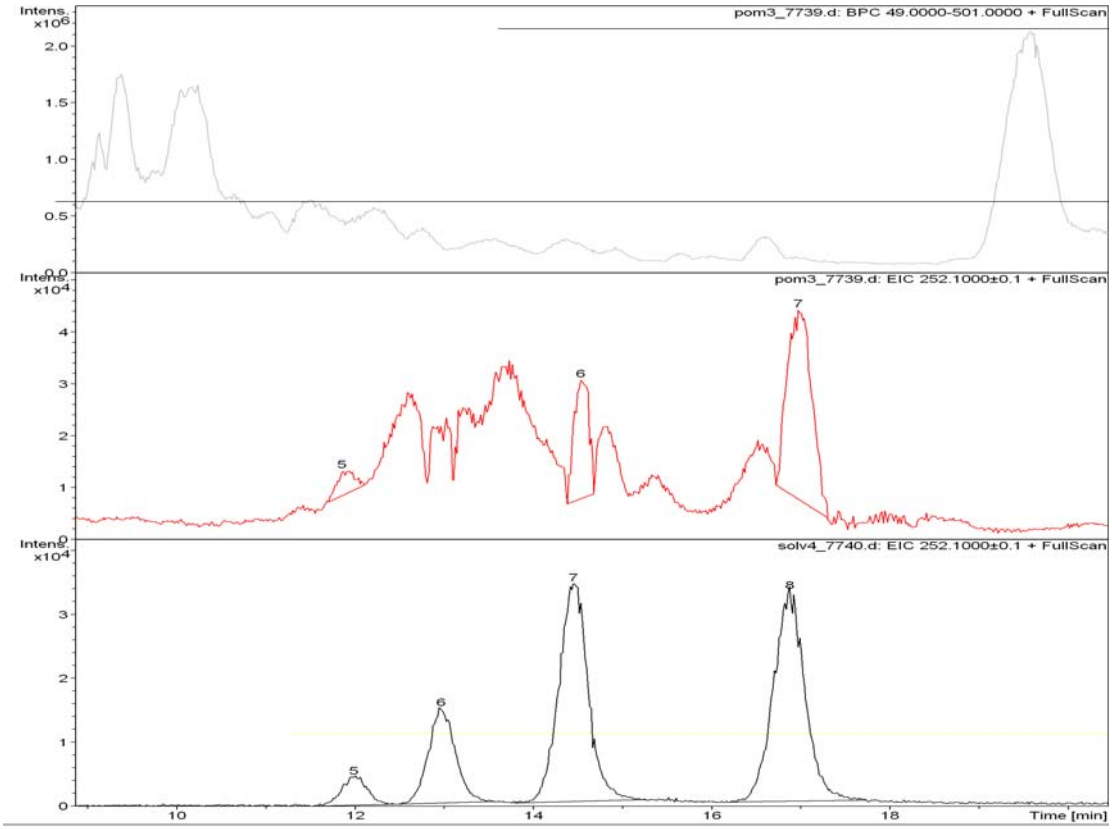
4.2. Standards in extract from Olive Pomace Oil

The picture is different for the instrument calibration with standards in olive pomace oil extract, mainly because detection of the target analytes by HPLC-FLD was not possible. Here the APLI analysis showed a clear advantage by detecting 8 of the 16 analytes. However, the sensitivity and precision decreased significantly compared to the measurements of the standard solutions in solvent. It was questionable wether the provisions given in EU legislation could be met with that technique, presuming analyte enrichment factors of less or equal to ten [25].

As an example, an extract of an HPLC-APLI-MS chromatogram of a spiked olive pomace oil extract is shown in Figure 1 for the retention time window between 9 and 20 min. The chromatogram at the top of Figure 1 displays the total ion chromatogram of the mass spectrometric scan from m/z=50 to m/z=500. In the middle of Figure 1 the single ion chromatogram of m/z=252 is presented for this sample. This mass-to-charge ratio corresponds to the molecular ion mass of the three benzofluoranthenes as well as to that of BaP. The peaks of these four substances in a standard solution are displayed as a reference in the single ion chromatogram (m/z=252) at the bottom of Figure 1. The elution order was BjF (12.1 min), BbF (13.0 min), BkF (14.5 min), and BaP (17.1 min). The lower two chromatograms in Figure 1 have the same abundance scaling, whereas the peak intensity of the largest peak in the top chromatogram was about 100 times larger than that of the single ion chromatograms.

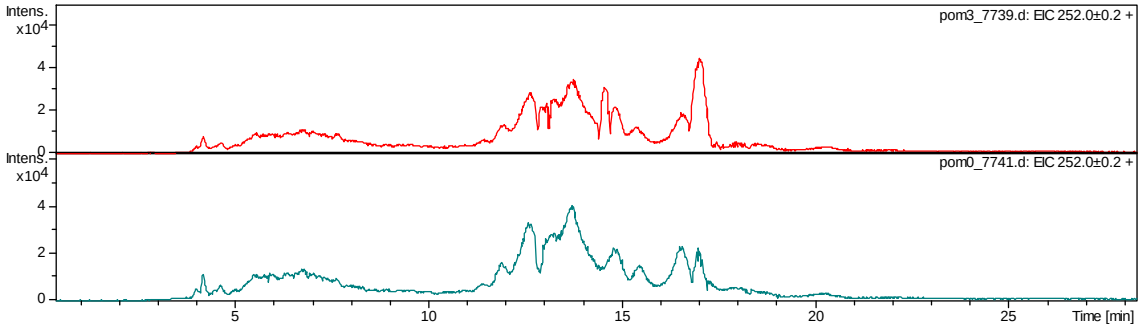
From Figure 1 it may be concluded that neither unambiguous peak identification, nor accurate quantitation was possible for this matrix matched calibration standard. Only three of the four PAHs that are expected in this retention time window having this m/z-value might have been detected in the olive pomace oil extract. However, high uncertainty remains with the identification of the analytes due to incomplete chromatographic resolution. Benzo[*b*]fluoranthene, which is expected to be of high relevance for official food control in future, cannot be identified at all, despite a content of 4.3 µg/kg. The total ion chromatogram of the olive pomace oil sample shows at the retention time of the four analytes in question much lower signal intensities than at the begin and at the end of the retention time window. Hence matrix interferences are even more likely in this retention time areas.

Figure 1: Extract of the LC-APLI-MS chromatograms of a matrix matched calibration standard. Retention time range displayed: 9 min to 20 min; top: total ion chromatogram of the MS scan over the range m/z=50 to m/z=500;
middle: single ion chromatogram for m/z=252 with a mass resolution of 0.1 m/z
bottom: single ion chromatogram for m/z=252 of a standard solution in ACN, mass resolution was 0.1 m/z



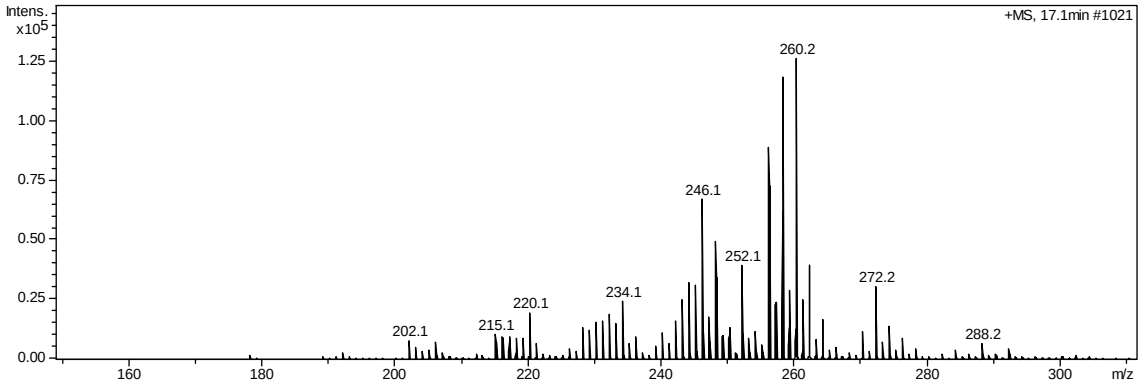
Most of the peaks recorded for the spiked olive pomace oil were also present in the HPLC-APLI-MS chromatogram of the native (not spiked) olive pomace oil. Figure 2 presents the single ion chromatograms for the mass-to-charge ratio 252 of the spiked olive pomace oil (upper chromatogram, spiking level 4.3 µg/kg for each analyte) and of the native olive pomace oil (lower chromatogram). When comparing the two chromatograms it even seems that some analytes were already present in the native olive pomace oil, e.g. BaP which elutes at 17.1 minutes (high peak in upper chromatogram)

Figure 2: Single ion chromatograms for m/z=252 of the spiked olive pomace oil (upper chromatogram) and the native olive pomace oil (lower chromatogram)



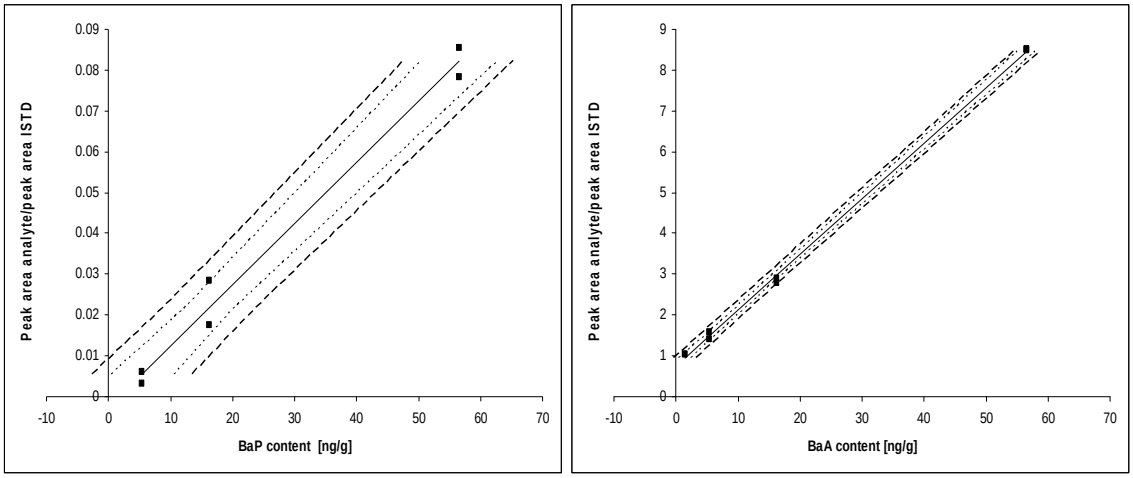
However, the spectrum of the peak at 17.1 min of the spiked olive pomace oil (displayed in Figure 3) shows a large number of mass peaks. The base peak ion of BaP (m/z=252.1) is surrounded by more abundant ions which seem to belong to a homologous series.

Figure 3: Mass spectrum of peak at the retention time of BaP in the spiked olive pomace oil.



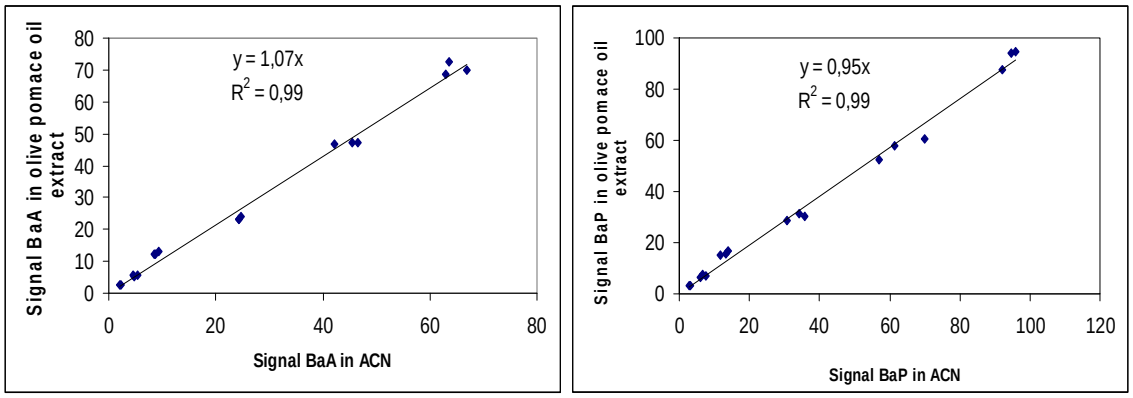
The matrix interferences provided by the olive pomace oil were not equal for all analytes as can be seen in the two calibration curves depicted in Figure 4. The confidence and prediction intervals were much broader for BaP (left side of Figure 4) than for BaA (right side of Figure 4), which is the consequence of lower agreement between the measurement values and the regression curve. Noticeably only the three highest concentration levels of BaP could be distinguished in the matrix matched calibration from the background. The analyte content of the lowest calibration point was for BaA even about four times lower than the content of the respective calibration level for BaP, confirming both the strong influences of the matrix on the BaP peak and the large differences of matrix interferences experienced by the different target analytes.

Figure 4: Calibration curves for BaP and BaA with confidence and prediction intervals (both at the 95 % confidence level) obtained from thr measurements of matrix matched calibration standards



The measurement of the matrix matched calibration standards by HPLC-DA-APPI-MS were much less affected by matrix interferences. As an example Figure 5 shows for BaP and BaA plots of the signal intensities of the measurements of the standard solutions in acetonitrile plotted against the signal intensities for the respective analyte in the measurements of the matrix matched calibration standards. In both cases the slope of the regression curve is close to one, which indicates the absence of matrix effects.

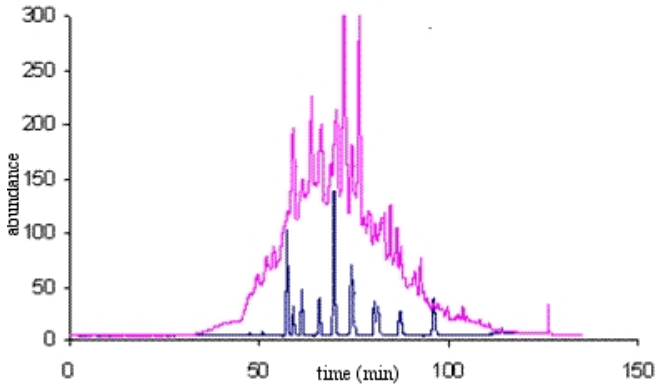
Figure 5: Plots of the signal intensities of the measurements of standards in ACN against the respective signal intensities of matrix matched calibration standards: left side: BaP, right side BaA



4.3. Test material of FAPAS round 0630 and BCR-458

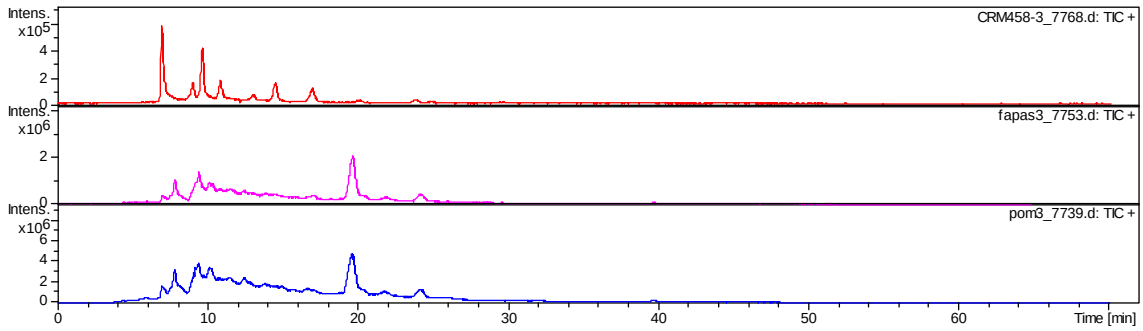
The test material of FAPAS proficiency test round 0630 consists of a very complex matrix. The determination of the 15+1 EU priority PAHs in that material was not possible by HPLC-FLD after sample preparation by DACC. Figure 6 shows a HPLC-FLD chromatogram (excitation wavelength 270 nm, emission wavelength 420 nm) of that sample (in pink) and at the bottom as a reference the chromatogram of CRM 459 (blank coconut oil) spiked with the respective analytes to a level of 5.0 µg/kg each.

Figure 6: Test sample of FAPAS® round 0630 analysed by HPLC-FLD after sample clean up by DACC



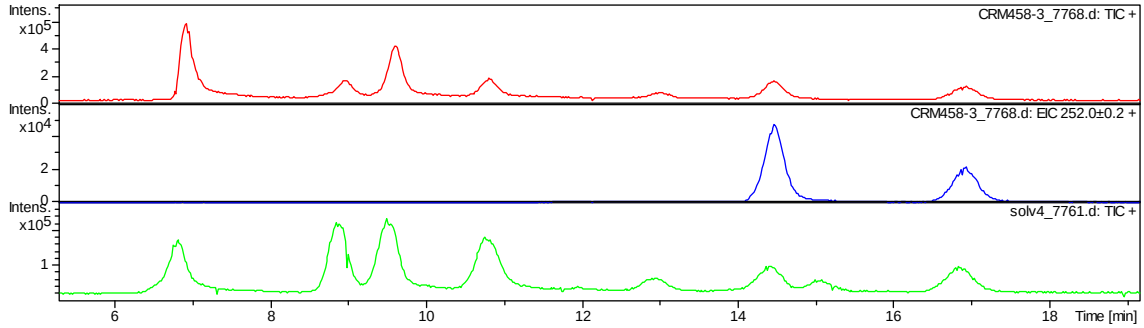
The HPLC-APLI-MS chromatograms of the FAPAS® sample were similar to the chromatograms gained for the matrix matched calibration standards, which means that unambiguous peak identification and accurate quantification was not possible for some analytes due to strong matrix interferences. Figure 7 shows the total ion chromatograms of BCR-458 (at the top), of the FAPAS® round 0630 sample (in the middle), and of the spiked olive pomace oil sample (at the bottom). The chromatograms of the two olive pomace oil samples (lower two) are nearly identical; dispite different origin of the native olive pomace oil is very likely. This underpins the assumption that the experienced matrix interferences are characteristic for this particular type of edible oil. Contrary to that, the chromatogram of the BCR-458 certified reference material did not indicate severe matrix interferences. All target analytes could be easily identified and integrated, as demonstrated in Figure 8.

Figure 7: Total ion chromatograms of HPLC-APLI-MS measurements of BCR-458 (at the top), FAPAS® round 0630 sample (in the middle), and the matrix matched calibration standard (at the bottom, spiking level: 4.3 µg/kg for each target analyte)



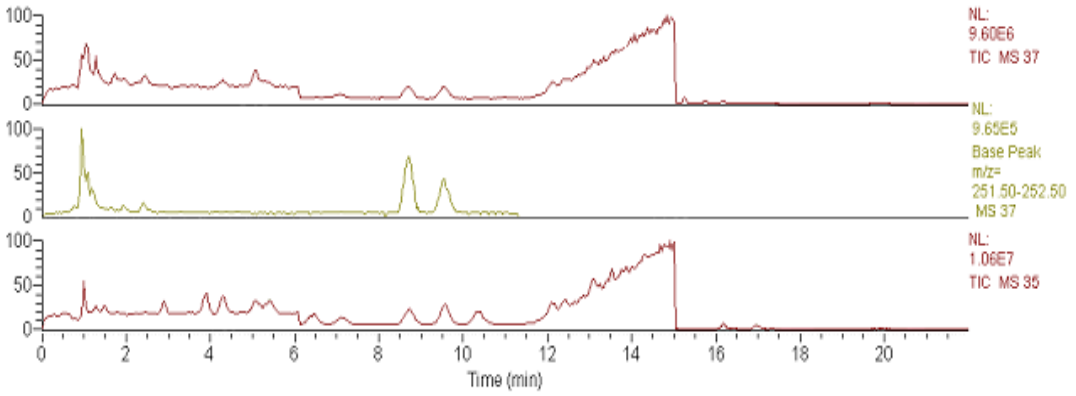
The chromatogram of the standard solution in ACN serves in Figure 8 as reference for peak identfication. The single ion chromatogram of m/z=252 contains only two peaks representing BkF and BaP, which is very different to the respective single ion chromatograms obtained for the olive pomace oils. The overall profiles of the chromatogram of BCR-458 and of the standard solution in ACN agree very well, except that peak hights are varying due to differences in the analyte contents.

Figure 8: HPLC-APLI-MS chromatograms of BCR-458 and a PAH standard solution in ACN; at the top: total ion chromatogram, in the middle: single ion chromatogram of m/z=252, at the bottom: total ion chromatogram of PAH standard solution in ACN



The corresponding chromatograms obtained in the measurments of BCR-458 by HPLC-DA-APPI-MS are displayed in Figure 9. Differences in retention times compared to Figure 8 are caused by different gradients of the mobile phase. However, the single ion peaks for BkF and BaP (Figure 9, in the middle) are, as in Figure 8, well separated and due to the absence of baseline distortions easy to integrate.

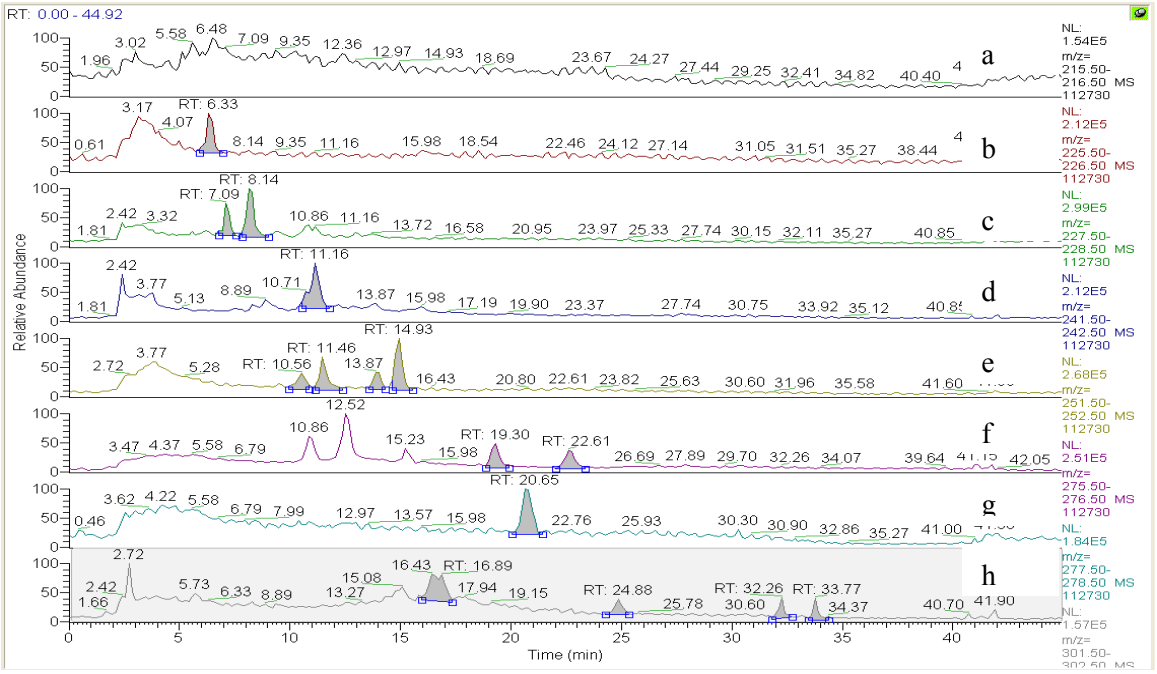
Figure 9: HPLC-DA-APPI-MS chromatograms of BCR-458 and a PAH standard solution in ACN; at the top: total ion chromatogram, in the middle: single ion chromatogram of m/z=252, at the bottom: total ion chromatogram of PAH standard solution in ACN



The single ion chromatograms recorded for the sample of FAPAS® round 0630 are displayed in Figure 10. Most chromatograms contain a smooth baseline, which indicates a lack of isobaric interferences. Only the peaks of 5-MC (Figure 10 d) and DlP (Figure 10 h) are interfered by other substances. The content of BcL was in this sample 2.1 µg/kg, which was below the detection

limit. Remarkable was the ability to determine CPP at an assigned level of 2.3 µg/kg (Figure 10 b), which cannot be reached with any other HPLC based analysis method.

Figure 10: Single ion chromatograms of the sample of FAPAS® round 0630 analysed by HPLC-DA-APPI-MS
a: BcL; **b**: CPP; **c**: BaA, CHR; **d**: 5-MC; **e**: BjF, BbF, BkF, BaP; **f**: BgP, IcP; **g**: DhA; **h**: DlP, DeP, DiP, DhP



5. CONCLUSIONS

The project aimed to evaluate the performance of three HPLC-detection methods for the determination of the 15+1 EU priority polycyclic aromatic hydrocarbons (PAHs) in solvent solution and model food extracts. The detection methods were fluorescence detection (FLD), atmospheric pressure laser ionisation mass spectrometry (APLI-MS), and dopant assisted atmospheric pressure photoionisation mass spectrometry (DA-APPI-MS). The latter two techniques are rather new and information on their performance in the analysis of food samples has not been published yet.

Donor acceptor complex chromatography (DACC) was applied for the preparation of all food matrix samples included in the experiments.

In general, the FLD measurements of standard solutions in acetonitrile gave for the majority of target analytes lowest values of the instrument detection limit, which were determined according to ISO Standard 11843-2:2000. Cyclopenta[*cd*]pyrene was not at all detectable either by FLD or APLI-MS, which indicates a common step in the excitation respectively ionisation process of this compound. The gap of non-detectability of CPP can be closed by application of DA-APPI-MS, which is able to detect this substance with acceptable sensitivity.

Many problems were experienced in the analysis of spiked olive pomace oil extracts. Matrix interferences made the quantification of most target compounds in the FLD and APLI-MS

chromatograms impossible. Low chromatographic background and sufficient sensitivity was only achieved for this type of matrix by applying DA-APPI-MS. All but one compound could be baseline integrated. Precision of replicate measurements was high, and the detection system was stable for at least 50 injections. However it has to be mentioned that the HPLC-APLI-MS analysis method was not optimised due to time constraints.

A possibility to circumvent the problems experienced in direct APLI ionisation of the target PAHs could be provided by application of a dopant also in the APLI measurements. Such dopant assisted APLI-MS might allow combining the high energy flux of the laser with the charge/proton transfer ionisation mechanism provided by the dopant. First steps into this direction were recently made at the Institute for Physical Chemistry of the University of Wuppertal (Germany), but only model compounds in very simple matrices were tested so far.

The next step in the development of HPLC-MS methods for the determination of EU priority PAHs in food could be the evaluation of the performance of dopant assisted laser ionisation mass spectrometry for this analysis task. However, this will depend on the accessability of suitable instrumentation and availability of sufficient man power.

6. ACKNOWLEDGEMENTS

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Food safety and quality

Development and optimization of a method for quantification of fishmeals in feeds by real-time PCR

Date: 15/05/2009
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Approved by: Franz Ulberth

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1. SUMMARY

Feedinstuffs containing processed animal proteins (PAPs) other than fishmeal can not be used in animal nutrition. Fishmeal is an authorised ingredient for pig and poultry feed even though is not used in practice due to cross-contamination problems. In order to facilitate the legal use of fishmeal in animal nutrition, the introduction of tolerance levels is considered. This is a new challenge requiring control tools to quantify accurately the level of PAPs in feed. This project intends to investigate in which way fishmeal can be detected and quantified in feedingstuffs by real-time PCR. Analysis of fishmeal needs a specific detection and quantification approach due to its high heterogeneity concerning species, place of origin, and tissues used.

In this exploratory research project, we have developed two real-time PCR methods for the quantification of fish DNA that could serve as indicator of the presence of fishmeals in feed. One method has been developed using the COX3/Gly/ND3 region in the mitochondrial DNA as target, while the other method has been developed using one region of the rhodopsin gene from the nuclear DNA. This would allow us to compare both methods and establish which target would be more appropriate for quantification of fishmeals.

2. INTRODUCTION

Since bovine spongiform encephalopathy (BSE) was reported for the first time in 1986 in the UK, the European Commission has developed a comprehensive set of risk reducing measures on transmissible spongiform encephalopathies (TSEs) in order to protect human health from BSE and to control and eventually eradicate TSEs in animals.

One of the most effective risk reducing measures consisted of an EU wide ban of PAPs are banned from use as feed ingredients for all farmed animals, with some exceptions (i.e. use of fishmeal in non-ruminants).

The use of PAPs is controlled within the European Union through several Regulations. Regulation (EC) No. 999/2001 (1) prohibits the feeding of mammalian proteins to ruminants, whereas Regulation (EC) No. 1774/2002 (2) introduced, among others, the ban of feeding animals with proteins from the same species (ban of intra-species recycling). Enforcing these regulations required analytical methods capable to allow species-specific identification. The lack of such methods led to the introduction of an extended feed ban for all farmed animals by amending Regulation (EC) No. 999/2001 through Commission Regulation (EC) No. 1234/2003 (3).

Though fishmeal is the only source of PAPs authorised for pig and poultry feed, it is not often used in practice because of the unavoidable occurrence of traces of fishmeal in ruminant feed by

non-intentional cross-contamination in feed mills. This would be expected in cases where feed for the various species are prepared on the site. The fact that there is no tolerance level for such traces poses severe problems to the risk manager. In order to facilitate the legal use of fishmeal in target feed, Regulation (EC) No 1923/2006 (4) introduces the possibility of a tolerance level for fishmeals (still unknown, but expected in the range of 1%), which requires for official control purposes the availability of a specific quantitative method.

2.1. Methods used for PAPs analysis

Classical microscopy is currently the only method officially endorsed by the European Commission to test for the presence of animal constituents in feeds. One of the restrictions of classical microscopy is the fact that the method has limited perspectives in terms of species-specific determination of PAPs. However, as stated in Directive 2003/126/EC (5), alternative methods can be used to gain more information about the origin of the found PAPs. Different methods have been developed to detect PAPs as well as to identify their origin at the species level, and their potential to detect PAPs in feedingstuffs at the benchmark level of 0.1% in feed has been demostrated; among them, the following methods are most often used: near infrared microscopy (NIRM); immunoassays and polymerase chain reaction (PCR).

Since PAPs represent a complex mixture of different substances and constituents, none of these methods measure PAPs "as such", but they detect specific "targets" indicating the presence of PAPs. In fact, classical microscopy and NIRM focus mainly on animal particles, whereas immunoassays and PCR measure specific proteins and DNA targets, respectively. In consequence, the quantification of the specific target does necessarily allow for the quantification of the PAPs, if the mass ratio between the measured target and the PAPs is unknown.

Qualitative detection of PAPs in feed: Classical microscopy is mainly based on the detection of bones. The method is capable of discriminating between fish and mammalian PAPs, but its sensitivity in detection of mammalian processed animal proteins decreases in the presence of significant amounts of fishmeal. The method has limited perspectives in terms of species-specific determination of PAPs. Near infrared microscopic methods are based on the use of the infrared spectra of individual particles to discriminate the origin of the feed compounds making up the samples. The discrimination of terrestrial PAPs from fish by-products can be accomplished by these methods but the technique can only give and indication about the species origin of the detected PAPs. An inter-laboratory study conducted by the IRMM (6) evaluated the performance profile of several real-time PCR methods. The results demonstrated sufficient sensitivity of these methods allowing the detection of cattle meat and bone meal in feed at 0.1% level even if fishmeal was present.

Quantitative determination of PAPs in feed: A recent interlaboratory study (7) evaluated the capability of classical microscopy to quantify fishmeal. The quantification requires the use of factors introducing sources of errors. The assessment revealed that there is some potential of the method for this purpose but there is still a high variability of results among laboratories, which makes this method not reliable for the purpose of quantification in the present format. The potential of classical microscopy regarding quantification of fishmeal at lower levels has not been evaluated yet. Regarding the quantification of PAPs by NIRM methods, several studies have shown their potential (8), nevertheless, the existing protocols – mainly developed for qualitative analysis- need further development in order to include mandatory steps for the quantification aspect.

Though real time PCR has in principle the potential of quantifying PAPs in feed(6), the application is complicated by the unknown process conditions of the PAPs to be quantified in feed. It is known that different temperatures applied during sterilisation have a strong impact on the integrity of the target DNA. This applies especially to mammalian PAPs, but less strong to fishmeals, which are treated at lower temperatures compared to other animal meals. This makes fishmeals a very interesting material for investigating the possibility of using real-time PCR as a quantitative technique for this material in feeds.

Quantitative real-time PCR has been successfully applied for animal species identification in food and to a minor extent, in feed (10, 11), but for the moment there is little information for its application for fishmeal quantification in feeds.

On the other side, different approaches are being followed by different research groups working on the development of such methods concerning target DNA. Some methods are developed for the detection of mitochondrial DNA, whose presence in multiple copies per cell allows detection even in highly treated samples. Other methods focus on single copy nuclear DNA, less abundant but more feasible for quantification due to its more constant copy number in different cells from different tissues. More profound studies are needed to establish appropriate targets for DNA quantification in feeds.

Our main propose was to develop two different real-time PCR methods for quantification of fishmeals in feed, one method based on a nuclear DNA target and a second method based on mitochondrial DNA target, to compare both methods and to check which would be more suitable for our objective.

2.2. Characteristics of fishmeals

Fishmeal is the brown flour obtained after cooking, pressing, drying and milling whole fish and food fish trimmings. Use of whole fish is almost exclusively from small, bony species of pelagic

fish (generally living in the surface waters or middle depth of the sea), this fish exclusively captured for the production of fishmeal represents around 67% of fishmeal production and it includes 7 different fish species, the 33% left is composed of trimming from food industry, thus including the fish species typically included in the human diet (16). All fish species captured exclusively for fishmeal production and most of the species used by the food industry, belong to three different orders: salmoniformes, clupeiformes and perciformes, which considerably reduces the possible genetic variability among them (17).

DNA is a quite thermostable molecule, however it has been reported that intensive treatments, such as those applied to certain animal meals, might affect the integrity of DNA and hinder its detection by PCR (12). The use of small DNA targets have shown to be a key issue for animal meals detection in feed (9). Quantification is at the moment not possible, due to the difficulty of knowing which sterilization process was applied to these meals and how this affected the integrity of the DNA. In addition, the *unknown* variability of animal meals, in terms of their composition, might as well affect the amount of amplificable DNA. Both issues would affect the integrity and the amount of animal DNA present on the sample, which makes the relation between DNA copy number and percentage of animal meal highly variable.

Concerning the processing of fishmeals and contrary to other meat meals, the heat treatment typically does not pass 90°C. This would affect much less the integrity of the DNA, and at the same time, the use of whole fish can compensate possible differences of DNA content between different tissues. As reported here, fishmeals have specific characteristics that might affect the development of quantitative methods.

2.3. Main objectives of the study

The main objectives of this study were, in the first place, to elaborate a database of possible mitochondrial and nuclear DNA targets to detect fishmeals. Once the database was established, the next objective was to design two different real-time PCR methods: (1) a method based on mitochondrial DNA, developed in CRA-W and (2) a method based on nuclear DNA developed in IRMM. The last objective was to test them in well-characterized fish species to see possible differences among Ct values from each of them.

2.4. Strategy for method development

A database was elaborated by DNA sequence searching in public databases and scientific literature. Several target sequences were analysed in order to choose the most appropriate.

Well-characterised fish species were necessary to test the methods. Different research institutes were contacted, and different fish species were acquired from the Max Rubner Federal Research Centre for Nutrition and Food-Department of Fish Quality (Hamburg, Germany). Nine different hake species were kindly donated by the Marine Research Institute (IIM) from the Spanish National Research Council (CSIC) (Vigo, Spain). A complete list of fish species can be found in **annex 1**. In total, the sample bank consisted of 38 different fish samples corresponding to 27 different fish species, among them the 7 fish species usually captured for fishmeal production and some of the most important fish species usually used by the food industry.

2.4.1. Mitochondrial DNA method

For mitochondrial DNA the complete sequence is available for different animal species, which allows their alignment and comparison to search the region of interest. Few different fish species such as salmon, cod, trout and carp were aligned to look for conserved regions and compared with cat, dog, cattle, sheep, horse, donkey, pig, rabbit, human, chicken, ostrich and crow. The comparison allowed us to get a broad overview of the sequences of different animal species and to check also if the conserved regions were just conserved among fish and would not give cross reactivity with other animal species. The region of interest was identified at the COX3/Gly/ND3 (COX3 = Cytochrome Oxydase subunit III; Gly = tRNA-Gly ; ND3 = NADH dehydrogenase subunit 3). A pair of primers and 2 different probes were designed in this region to give a fragment of amplification of 88 bp. A combination of two probes was used to cover a bigger variety of fish species, since the internal sequence might have some variation in some species leading to miss amplification.

The method was tested with different fish species. For each sample a piece of flesh was dried with a SpeedVac. DNA extraction and purification was performed using a phenol/chloroform protocol (starting from a test portion of 200 mg). The DNA concentration was measured with a NanoDrop.

The DNA concentration was standardized at 10 ng/µl, in order to remove any PCR inhibition and to use the same DNA amount whatever the sample. This allowed us to check possible differences in Ct values according to the fish species. **Table 1** shows the corresponding Ct values obtained from different fish species with either of the probes (called fish and cod) and with the combination of both (called FISH in that case).

Species	FISH target	Cod target	Fish target
	Ct	Ct	Ct
Gadus morhua	24,29	25,17	24,7

Pollachius virens	25,79	27,79	27,14
Melanogrammus aeglefinus	25,03	25,35	24,32
Micromesistius poutassou	24,69	25,69	24,77
Sebastes spp. (marinus/mentella)	24,21	22,32	23,95
Mallotus villosus	38,34	41,56	37,14
Scomber scombrus	26,02	25,33	26,12
Clupea harengus	34,59	34,38	34,62
Merluccius merluccius	22,53	22,67	22,04
Trachurus trachurus	28,49	27,75	27,82
Trisopterus minutus	24,68	24,2	24,77
Sardina pilchardus	34,22	34,62	34,3
Engraulis encrasicolus	30,32	30,99	-
Gadus ogac	23,8	24,61	24,09
Trisopterus esmarki	25,51	26,11	26,1
Sand eel, species unknow	35,89	36,48	35,04
Sand eel, species unknow	35,56	36,28	34,66
Sprattus sprattus	>45.00	44,56	40,55
Hake	26,64	26,83	26,57
Hake	25,16	25,14	24,69
Negative control	50	50	50
Positive control	19,18	20,25	19,79

Table 1: Ct values obtained from DNA extracts from different well caracterised fish species (cod: just with "cod" probe, Fish: "fish" probe, FISH: combination of both probes).

As we can see in this table, there are small differences in the Ct values from the same species concerning the different probes used, but there are important differences concerning the different fish species (i.e. Ct values from 22 up to 45), which suggests that for fishmeals we would find similar results.

2.4.2. Nuclear DNA method

Concerning nuclear DNA, there are not so many DNA sequences available and not for the most important species of interest. Few possible targets were studied to investigate which would be the most suitable for the development of a real-time PCR method.

The possible targets investigated were alpha-actin, 5S ribosomal DNA (5S rDNA), preprogonadotropin-releasin hormone (GnRH), transferrin, growth hormone (GH) and rhodopsin genes (13, 14, 15). DNA sequence were investigated and obtained from public databases for these genes from the 27 species of interest.

According to the information available in the public databases three genes were selected to be further investigated, due to the higher number of sequences available in public databases (5S

rDNA, rodhopsin) or the existence of a real-time PCR method in scientific literature in the case of transferrin gene (13).

Concerning the 5SrDNA gene, this gene comprises a 120bp highly conserved coding sequence (5S rRNA) and a variable nontranscribed spacer (NTS). However the length of the NTS is usually species specific and the basic unit (5S rRNA + NTS) is tademly repeated a variable number of times on the chromosome depending of the species (14). This might affect the reliability of the quantification, since fishmeals can be a mixture of different species and their percentage in feed. In addition, in public databases it is possible to find sequences from the species of interests between 99 and 540 bp, but just for 9 of the species of interest the sequences available are larger than 150 bp, which would it make difficult to find an appropriate target for its quantification in all species of interest. The results from the alignment of the available sequences showed that the 5S rRNA fragment is well conserved among the different fish species, but also among other animal species, which indicates that it would not be an appropriate region to design fish specific primers. The NTS region, on the other side, is highly variable between fish species and other animal species, but also among fish species, which discards this region as well as possible target for our aim.

The transferrin gene codes an iron binding glycoprotein that plays a central role in the transport of iron between sites of absortion, storage and utilization in all vertebrate organisms (20). In fish, transferrin has an important role in the innate immune system, and the level of transferrin in blood is used as a health parameter (19). This gene was investigated since a method for the quantification by real-time PCR of haddock in commercial products using this target is available in the scientific literature (13). This method has shown to be able to quantify haddock in raw fish products. However, in public databases, only sequences from 2 out of 27 species of interest are available, which makes the comparison of the sequences problematic. Sequences from other fish species are available, and the gene map has been investigated in tilapia (20). As seen in **Figure 1**, the authors have identified conserved regions (exon-7 and exon-10) where they have designed PCR primers.

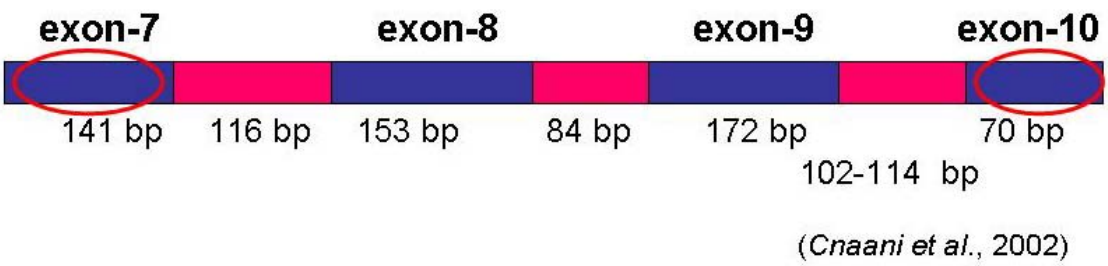

Figure 1: Part of the transferrin gene in tilapia.

These primers could be used to amplify DNA from the fish species of interest that would allow us to sequence and compare them in order to identify regions of interest to develop a real-time PCR method using these regions, which would allow us to quantify fish DNA in feed samples. However, the availability of sequences from just 2 out of the 27 species of interest makes it very difficult to evaluate the possibilities to find an appropriate region before starting the experimental work in the laboratory, which lead us to consider other possible target.

Rhodopsin is the membrane receptor responsible for photoreception in the vertebrate retina (21). The introless teleost fish rhodopsin gene (21) has been used in phylogenetic studies (23), and more recently has been used as a marker for genetic identification and characterisation of marine fish species from European waters and/or marketed in Europe in the Fish Trace European Project (24).

FishTrace provides information for the molecular identification of target species based on the sequences of the mitochondrial cytochrome b gene and the nuclear rhodopsin gene. Therefore, there is a lot of information available in public databases from this gene in different fish species. There were available, at the moment the search was performed, rhodopsin sequences from 16 out of the 27 species of interest. In addition, the fragment available is of 460 bp for all these species, which allows a better comparison and allows us to search for interesting regions inside this gene. From the alignment and analysis of these sequences with ClustaWl2 (http://www.ebi.ac.uk/Tools/clustalw2/), as seen in **annex 2**, we could confirm that there is no intraspecies variation, and regions with low interspecies variation between fish species and with high interespecies variation with no fish species. All this indicates that rhodopsin could be a good target for designing a real-time PCR method.

Samples were amplified following the approach described by Sevilla et al. (25). The authors suggest the use of nested polymerase chain reaction. Nested PCR involves two sets of primers used in two successive runs of the polymerase chain reaction; the second set is intended to amplify a secondary target within the amplicon of the first run, as seen in **Figure 2**.

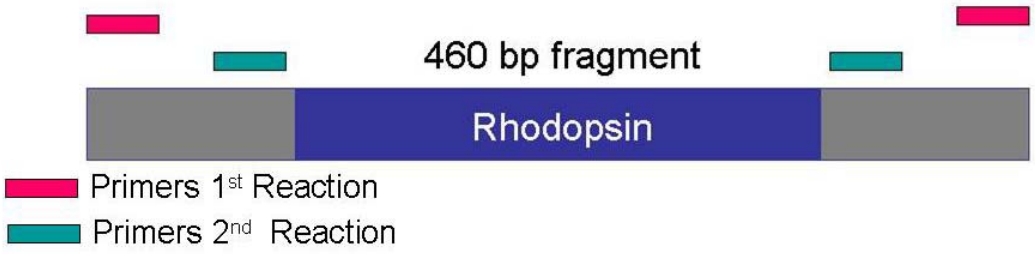

Figure 2: Nested PCR as proposed by Sevilla et al, for the amplification of a fragment of 460 bp of the rhodopsin gene.

This approach allows the obtention of a PCR product with higher purity. From the species of interest, 22 were successfully amplified and sequenced in collaboration with Dr. Eugénia de Andrade Silva from the Reference Materials Unit. The sequences were analysed with Geneious Pro 4.5.4. (26).

This software also allowed us to create a consensus sequence from all the fish species of interest. The procedure consisted of the alignment of all corrected sequences as seen in **Figure 3**, and from this alignment the consensus sequence was obtained, as seen in **Figure 4**.

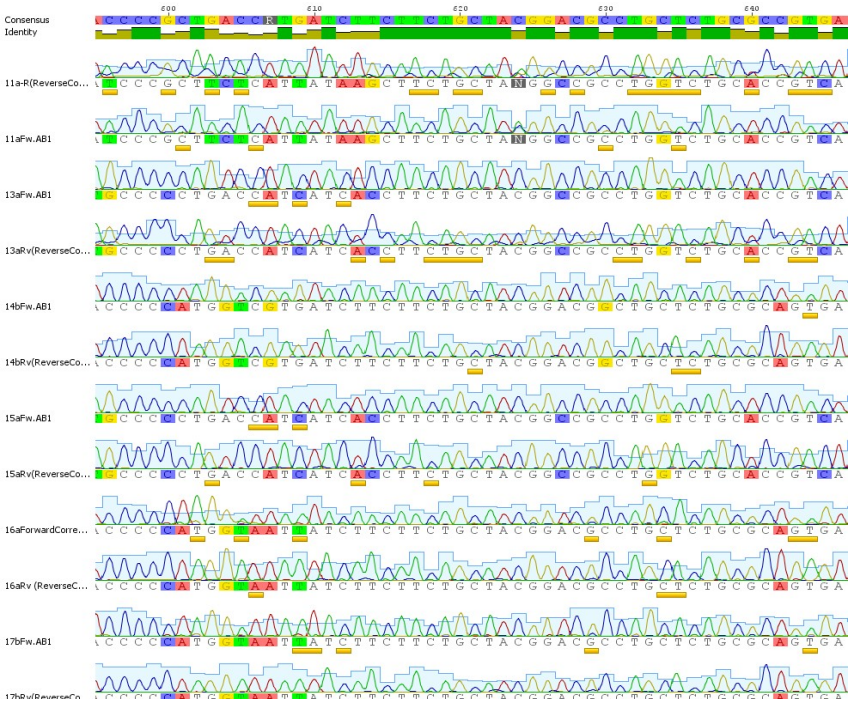

Figure 3: Alignment of fish species with Geneious Pro software.

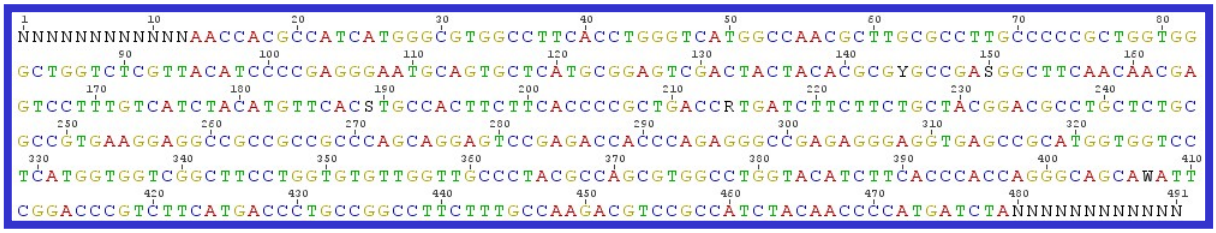

Figure 4: Consensus sequences obtained from all sequences from the different fish species by the Geneious Pro software.

This consensus sequence allowed us to develop a real-time PCR method for the quantification of DNA of any of the fish species of interest. Primers and probes for the qPCR assay were designed by using Primer Express software (Perkin Elmer Applied Biosystems), taking into account a few parameters to ensure the success of the method:

- The product of amplification should be between 50 and 120 bp. This would allow a positive signal and ensure the reliability of the quantification even if the heat treatment would affect the integrity of the DNA
- The PCR product should be in a well conserved region for all fish species of interest, which would be ensured by the fact that the primers and probes were designed from the consensus sequence
- Primers and probes should have mismatches with other species to avoid cross amplification that would lead to false positives and errors on quantification of fish DNA

From all the primers and probes suggested by the Primer Express software, one combination was chosen that would give a product of amplification of 87 bp. The consensus sequence was aligned with sequences for a broad variety of animal species such as cattle, human, rabbit and dog to ensure that they would not give a positive response with any other species apart form fish.

Figure 5: Alignments of consensus sequences with different animal species (primers and probe highlighted)

3. CONCLUSIONS

The initial objective of this project was to develop in parallel to different real-time PCR methods to quantify fishmeal in feeds. Therefore, both methods should be tested in well-characterised fish species and fishmeals. Due to inherent problems directly related with the study and also to a re-prioritisation of tasks, none of the methods could be tested in fishmeals and only the mitochondrial method was tested in raw fish. Primer and probes design was eventhough the crucial step in the project and the conclusions listed below demonstrate the importance of the achivements so far.

A mitochondrial DNA method has been developed by CRA-W and evaluated in well-characterised fish species. All fish species gave a positive reaction when the reaction mix included a combination of the two probes designed. However, there were important differences between the Ct values of the different species even if the same amount of total DNA was used. These differences might lead to inaccurate quantitative results for the estimation of fishmeals in feeds, since it is not possible to know the exact percentage of each fish species present as ingredient in the fishmeal.

In this case, the next step would be to test the method in different fishmeals to study the real effect on the reliability of the quantification.

A nuclear DNA method has been developed by IRMM. The in silico tests showed that the method seems promisingly fit for purpose. The next step would be (i) to test the method with well characterised fish samples, (ii) to study the Ct values for the different fish species and (iii) to test the method with different fishmeals to evaluate the possibility of quantifying them in feed.

Both methods should be compared and conclusions drawn, to determine which one is more suitable for the quantification of fishmeals in feed.

Twenty-two DNA sequences from different fish species will be published in the EMBL (European Molecular Biology Laboratory) database. Preliminary results were presented at the 1st European Food Conference in Ljubljana (Slovenia) in November 2008 and at the following events organised at the IRMM: "Life science symposium" on January 15th 2008, "Research fellows' seminar" on June 19th 2008 and at the "Exploratory research presentation of results" on May 13th 2009.

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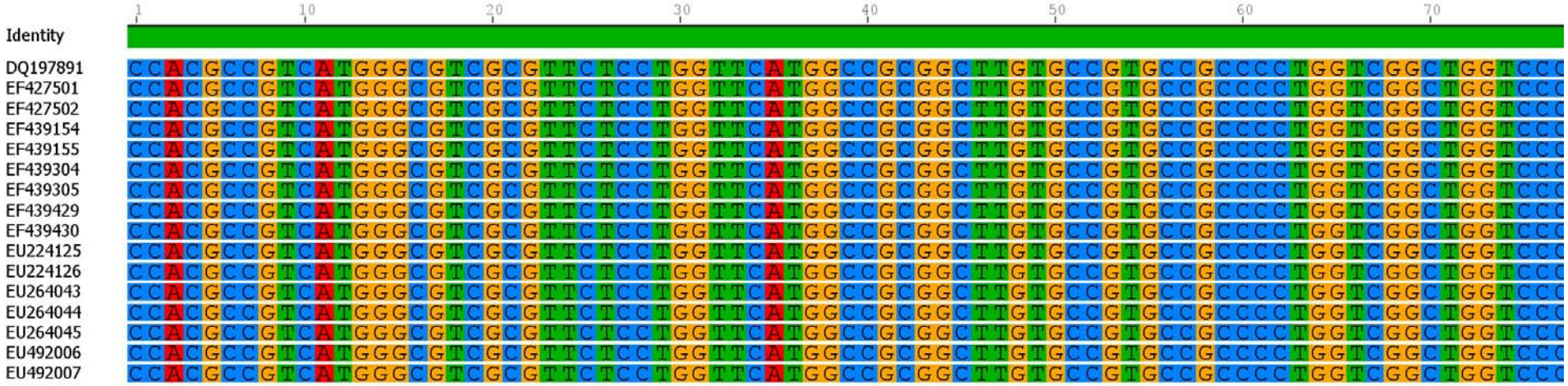
5. ANNEXES

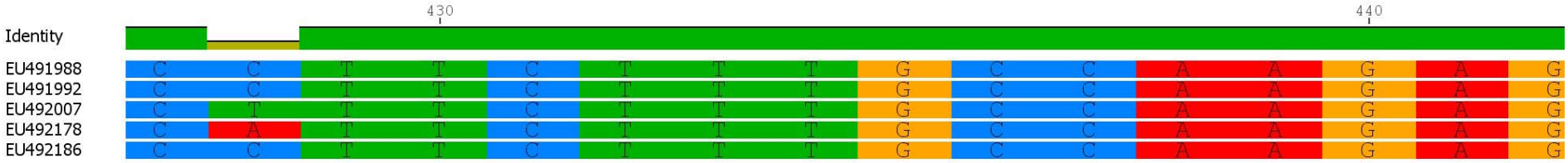
5.1. Annex 1: List of fish species

Common name	Sample Name	Samples codes	Origin
Well characterised species			
Norway pout	***Trisopterus esmarki***	G3001, G3012, G3016	BfEL (Hamburg, DE)
Pilchard	***Sardina pilchardus***	G3002, G3013, G3015	BfEL (Hamburg, DE)
Sprat	***Sprattus sprattus***	G3003, G3007, G3011	BfEL (Hamburg, DE)
Haddock	***Melanogrammus aeglefinus***	G3004,	BfEL (Hamburg, DE)
Saithe	***Pollachius virens***	G3005	BfEL (Hamburg, DE)
Mackerel	***Scomber scombrus***	G3006, G3019	BfEL (Hamburg, DE)
Hake	***Merluccius merluccius***	G3008	BfEL (Hamburg, DE)
		G3031	IIM (Vigo, ES)
Sand eel	**Sand eel**	G3009,G3023	BfEL (Hamburg, DE)
Herring	***Clupea harengus***	G3010, G3018	BfEL (Hamburg, DE)
Cod	***Gadus ogac***	G3014	BfEL (Hamburg, DE)
Blue whiting	***Micromesistius poutassau***	G3017	BfEL (Hamburg, DE)
Horse Mackerel	***Trachurus trachurus***	G3020	BfEL (Hamburg, DE)
Atlantic Cod	***Gadus morhua***	G3021	BfEL (Hamburg, DE)
Redfish	***Sebastes spp.***	G3022	BfEL (Hamburg, DE)
poor cod	***Trisopterus minutus***	G3024	BfEL (Hamburg, DE)
Capelin	***Mallotus villosus***	G3025, G3037	BfEL (Hamburg, DE)
Senegalese hake	***M. senegalensis***	G3028	IIM (Vigo, ES)
Southern hake	***M. australis***	G3029	IIM (Vigo, ES)
Blue grenadier	***Ma. Novazelandiae***	G3030	IIM (Vigo, ES)
Argentine hake	***M. hubbsi***	G3032	IIM (Vigo, ES)
Bengela hake	***M. Polli***	G3033	IIM (Vigo, ES)
South pacific hake	***M. gayi***	G3034	IIM (Vigo, ES)
Deep-water cape hake	***M. paradoxus***	G3035	IIM (Vigo, ES)
North pacific hake	***M. productus***	G3036	IIM (Vigo, ES)
European anchovy	***Engraulis encrasicolus***	G3038	BfEL (Hamburg, DE)
Additional species			
Yellowtail catfish	**Pangasiusfilet**	G3026	Local Supermarket (Delhaize)
Nile perch	**Victoriabaarsfilet**	G3027	Local Supermarket (Delhaize)

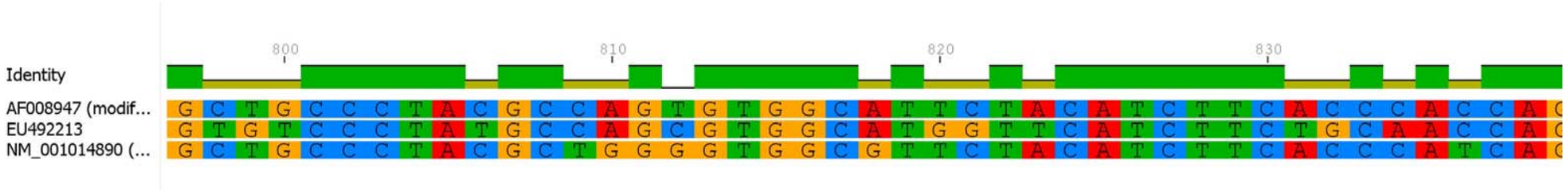
5.2. Annex 2: Alignments of rhodopsin sequences available in public databases



Detail of alignment of different sardine sequences showing no intraespecies variation



Detail of alignment of sequences from different fish species (anchovy, horse mackerel, mackerel, sprat and sardine) showing regions with low interespecies variation between fish species



Detail of alignment of sequences from different animal species (Norway pout against pork and cattle) showing regions with interspecies variation against "no fish" species

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Reference Materials and Measurements
Isotope measurements

Dual Isotope Analysis of Nitrate in Seawater, Fresh Water and Wastewater Using Chemical Reduction of N_2O

Date:	14/05/2009
Authors:	Angelika Nestler, Frederick Accoe, Michael Berglund, Lutgart van Nevel and Philip Taylor
Revised by:	Michael Berglund
Approved by:	

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1. SUMMARY

The aim of the exploratory research project is to develop a validated, fast and robust method for the analysis of the relative nitrogen and oxygen isotope-amount ratios ($\delta^{15}N$ and $\delta^{18}O$) of nitrate in seawater, fresh water and wastewater in support of the Water Framework Directive (1) and the recently published Marine Strategy Framework Directive (2).

The research approach of this project is based on the off-line chemical reduction of nitrate to N_2O in water samples (3). The water sample preparation includes two reduction steps: 1st reduction of nitrate to nitrite with zerovalent cadmium and 2nd reduction of nitrite to N_2O with a sodium azide/ acetic acid reagent (3, 4). Nitrous oxide serves as the analyte for the relative isotope-amount ratios. It is purged by a helium flow from the water samples into the online purification and pre-concentration peripheral (Finnigan™ GasBench II) of a continuous flow isotope ratio mass spectrometer (CF-IRMS, Delta V Advantage, Thermo) where the mass-to-charge ratios of 46/44 for $\delta^{18}O$ and 45/44 for $\delta^{15}N$ are analysed (5). Analytical precision of the method has been 0.23 to 0.63 ‰ for the $\delta^{18}O$ and 0.1 to 0.2 ‰ for $\delta^{15}N$ analysis. Trueness of the method has been proved by comparison of measurements of a working standard with the Gasbench II and Thermal Conversion / Elemental Analyser CF-IRMS.

A final evaluation of the method requires further investigations on the performance with sea water samples, surface water samples and other aqueous samples and as well further optimizations by reducing and quantification of blank effects, oxygen exchange with water and fractionation. An interlaboratory comparison with the method would be necessary to evaluate the method for surface water samples in comparison with the anion exchange method and with the bacterial method.

2. INTRODUCTION

2.1. Nitrate

Despite the efforts in the European community legislation nitrate (NO_3^-) is still a major pollutant and one of the nutrients causing eutrophication of surface waters and marine waters in the European Union (EU). The extent of eutrophication in the European Union is visble by chlorophyll-a concentration > 1 $mg \cdot L^{-1}$due to phytoplankton development (Figure 1).

Several European Directives address to reduce nitrate and other nutrient discharges and emissions into the environment:

- Nitrates Directive (91/676/EEC) (6),
- Urban Waste Water Treatment Directive (91/271/EEC) (7),
- The Water Framework Directive (2000/60/EEC) (1),
- Marine Strategy Framework Directive (2008/56/EC) (2).

The Nitrate Directive 91/676/EEC aims to reduce and prevent the pollution of water bodies caused by the application and storage of inorganic and organic fertilizers in agriculture. The objective is on the one hand to safeguard drinking water supplies and on the other hand to prevent eutrophication of freshwater and marine waters. The European Member States are obliged to monitor nitrate concentrations in surface and water against a maximum limit of 50 mg $NO_3^- \cdot L^{-1}$. Water bodies which exceed or tend to exceed this level must be designated as a Nitrate Vulnerable Zone (NVZ).

The Water Framework Directive (2000/60/EEC) (1) provides that for each river basin a river basin management plan should be formulated with an integration of ecological and chemical protection of water bodies and requires that all EU waters should achieve good status by 2015.

The objectives of the Marine Strategy Framework Directive (2008/56/EC) (2) are in line with the Water Framework Directive (2000/60/EEC) (1) and the aim is to achieve good environmental status of the EU's marine waters by 2021. Each EU Member State with marine water needs to develop marine strategies with an assessment of the state of the environment, a definition of "good environmental status" at regional level and the definition of environmental targets and the establishment of monitoring programmes.

In order to take source-oriented remedial actions for restoration of coastal and estuarine ecological status, apportionment of point or diffuse nitrate sources is essential.

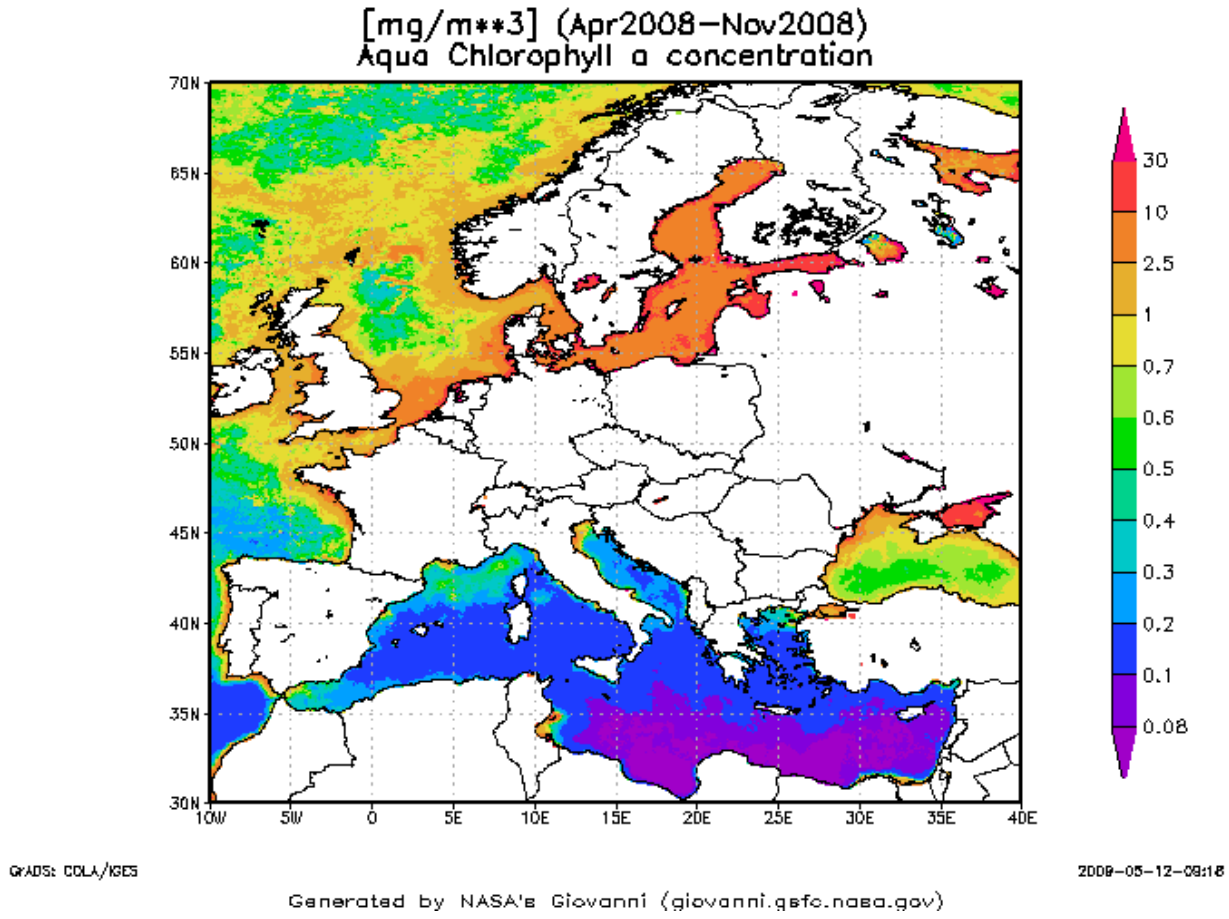

Figure 1: Satellite image of chlorophyll-a concentrations in marine ecosystems of the EU.

2.2. Nitrate source apportionment with stable isotopes

Several studies have demonstrated that analysis of the N and O isotopic composition of nitrate can be used as a powerful tool for identifying the sources of nitrate in surface water and groundwater (8-12). This is based on the distinct and characteristic N and O isotopic compositions for nitrates originating from different pollution sources (animal manure, mineral fertilizers, sewage, industrial wastewater, soil N, atmospheric deposition). Recently, a new method has been reported that enables dual isotope analysis (N and O) of nitrate in seawater (3). Some recent studies show that the dual isotope analysis can be successfully used as a tool to identify multiple nitrate sources and to study spatial mixing patterns of the nitrate pollution in coastal waters and estuaries (13, 14). In addition, the dual isotope analysis of nitrate could potentially be used to identify areas where natural nitrate attenuation processes (e.g. denitrification and phytoplankton uptake) occur, since these processes cause isotope fractionation which is reflected in the isotopic composition of the remaining nitrate pool (15).

2.3. Methods for the analysis of $\delta^{15}N$ and $\delta^{18}O$ in nitrate water samples

Methods for the analysis of $\delta^{15}N$ and $\delta^{18}O$ in nitrate water samples are given in Table 1. Since the N and O isotopic signature of nitrate can give information on both the sources and on natural attenuation processes, there is a need for a robust, validated method for dual isotope analysis of nitrate in seawater. The classical method for dual isotope analysis of nitrate in fresh water is based on pre-concentration of the nitrate on anion exchange resins, purification and conversion to silver nitrate ($AgNO_3$) for on-line TC/EA IRMS (Thermal Conversion/Elemental Analyser Isotope Ratio Mass Spectrometry) analysis (16). This method is labour-intensive and strongly affected by matrix interferences in samples with high ionic strengths or high contents of dissolved organics, which makes this method not applicable to seawater or nitrate source samples (e.g. sewage, industrial wastewater, animal manure, soil extract).

Both methods, the bacterial method and chemical reduction method, consist of an off-line chemical reduction of nitrate to nitrous oxide. The extraction, purification and pre-concentration of N_2O are realized by an on-line purge and trap system, followed by CF-IRMS analysis.

Table 1: Methods for the analysis of $\delta^{15}N$ and $\delta^{18}O$ in nitrate water samples

Method	Anion exchange[1]	Bacterial[2,3]	Chemical[4]
analyte	$AgNO_3$	N_2O	N_2O
analysis	TC/EA IRMS	GasBench / IRMS	GasBench / IRMS
oxygen exchange with water	no	yes, mathematical approach needed	acidic pH, correction
fractionation	minimal	yes, mathematical approach needed	correction
interferences	high anion concentrations	toxic substances, nitrite, NO	(nitrite)
precision $\delta^{15}N$ (1 σ)	0.05 ‰	0.2 ‰	0.2 ‰
precision $\delta^{18}O$ (1 σ)	0.5 ‰	0.34 ‰ to 0.95%	0.5 ‰
concentration	1 µmol·L^{-1}	1 µmol·L^{-1}	0.5 µmol·L^{-1}
time / costs material	3-5 d, 60 €	2-3 d (+10 d) , 5 €	1.5 d, ~ 5 €

[1] (16) [2] (17) [3] (5) [4] (3)

3. MATERIAL AND METHOD

3.1. Experimental set up

The experimental set up for the analysis of the isotopic composition of nitrate is illustrated in Figure 2. Fractionation, oxygen exchange and blank effects can differ with each batch of activated cadmium and as well with each batch of produced sodium azide / acetic acid solution. Therefore the standardisation of the isotopic analysis has to be realised for each experimental set up. Thus, this requires including the isotopic standards in each experimental set up and they undergo the same sample preparation procedure as the sample replicates and the quality assurance (QA) sample replicate.

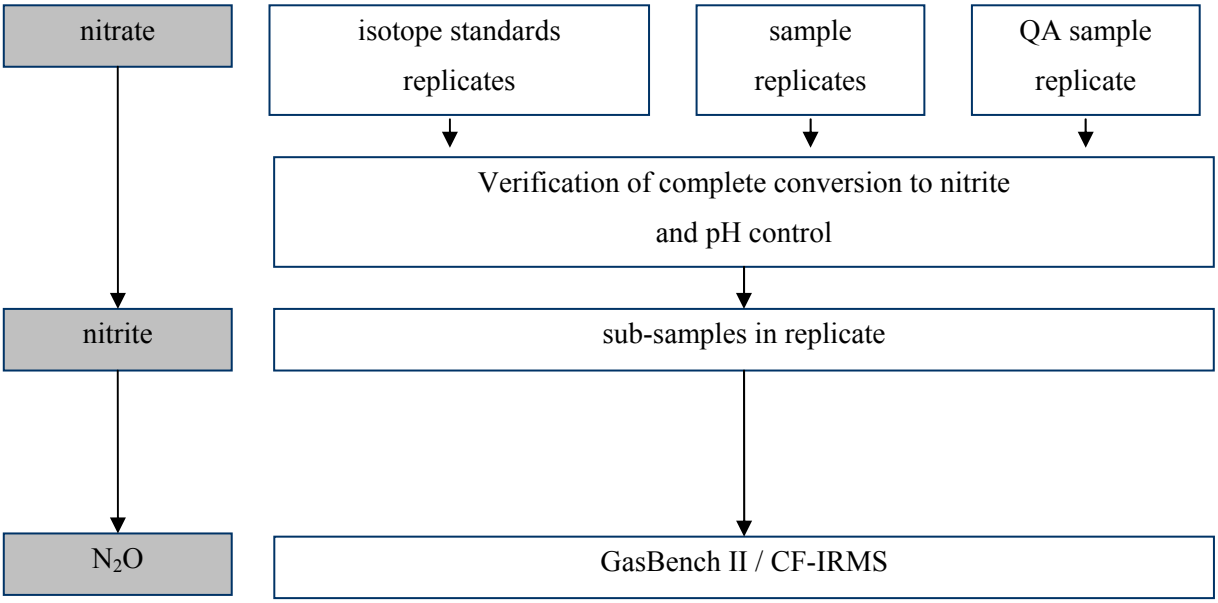

Figure 2: Experimental set up for the analysis of $\delta^{15}N$ and $\delta^{18}O$ in nitrate water samples with the chemical reduction method according to (3).

3.2. Potassium nitrate working standard and nitrate isotopic standards

Potassium nitrate (Merck, Darmstadt) is used as a working standard for the method evaluation. Measurements of $\delta^{18}O$ and $\delta^{15}N$ values with the TC/EA CF-IRMS are executed as described in (18).

Solutions with a nominal concentration of 1 mmol·L^{-1} nominal concentrations are produced with the nitrate working standard (potassium nitrate, Merck Darmstadt) and the nitrate isotope standards USGS 34, USGS 35 and IAEA-N3 (Table 2). This solutions are used to produce an artificial sample of 20 µmol·L^{-1} and as well the isotope standard solutions. Therefore 0.5 mL of the 1 mmol·L^{-1} nitrate solutions are diluted in 24.5 mL 0.5 M sodium chloride solution.

Table 2: Reference values for the relative N and O isotope-amount ratios of the nitrate reference materials (19, 20)

isotope standard	chemical formular	relative isotope-amount ratios	NIST (20)		Brand et al. (19)	
			value	U	value	U
IAEA-N3	KNO_3	$\delta^{15}N_{AIR} \cdot 10^3$	4.7	± 0.3		
		$\delta^{18}O_{VSMOW} \cdot 10^3$	25.6	± 0.4	25.32	± 0.58
USGS 34	KNO_3	$\delta^{15}N_{AIR} \cdot 10^3$	-1.8	± 0.2		
		$\delta^{18}O_{VSMOW} \cdot 10^3$	-27.9	± 0.6	-27.78	± 0.74
USGS 35	$NaNO_3$	$\delta^{15}N_{AIR} \cdot 10^3$	2.7	± 0.2		
		$\delta^{18}O_{VSMOW} \cdot 10^3$	57.5	± 0.4	56.81	± 0.62

3.3. Sample preparation

3.3.1. Precautionary measures for the sample preparation

Since the cadmium and the sodium azide are both categorised as very toxic and as dangerous for the environment comprehensive safety, health and environmental precautionary measures are required for this method. During both sample preparation steps the risk of inhalation of very toxic particles or very volatile substances (hydrozoic acid, which evolves during the sodium azide / acetic acide reduction step) is given. All manipulations of the cadmium and sodium azide and as well with the other chemicals require personal protective equipment (proper gloves, bracer, googles, and laboratory coat) and a properly working fume hood or a glove box in order to minimize the risk as low as possible. Solutions of sodium azide waste needs to be collected separately as hazardous waste under basic conditions to consume all remaining hydrozoic acid. The other solid and liquid chemical wastes also need to be collected as hazardous waste.

3.3.2. Conversion of nitrate to nitrite with zerovalent cadmium

The first reduction step of NO_3^- to NO_2^- by cadmium has usually been applied for the spectrometric analysis of nitrate in water (Morris and Riley, 1963; Wood et al., 1967; Margeson et al., 1980). The reduction of nitrate to nitrite in waters samples takes places in Teflon sealed batches, shaking overnight (horizontal, 120 rpm), containing 50 mL water sample, 1 g spongy cadmium buffered with 1M imidazole solution to pH 9 (3). Freshwater samples require a sodium chloride concentration of 0.5 M. After separation of the spongy cadmium by centrifugation the water samples are introduced in new vials. This reduction step is altered by Schilman and Teplyakov (21) using a cooperized granular-cadmium filled-column. When the sample passes with a flow rate of 5 mL $\cdot$ min^{-1} through the column a complete conversion of nitrate to nitrite avoiding any isotopic fractionation can be achieved within a few minutes.

The column method of (21) has been tested as well for the reduction to of nitrate but due to the observed memory effects and incomplete conversion of nitrate it has not been further included in the investigation. Also Altabet (22) has been investigated this method and observed huge deviations of the results between of two columns.

The method of McIlvin and Altabet (3) has been optimised by Altabet (4) and the protocol was provided to IRMM for this exploratory research project. In this optimized method powdered cadmium (Alfa Aesar, mesh -325) is used instead of the spongy cadmium. For the activation, the powdered cadmium is covered with a few drops deionized water (MilliQ) and then hydrochloric acid (5%) is added drop wise until the colour turn from black to metal grey, afterwards the cadmium is rinsed until it is neutral (23). Water samples (25 mL) containing 20 µmol$\cdot L^{-1}$ nitrate in 0.5M sodium chloride were filled in centrifuge tubes. To all samples, 0.25 mL 1 M imidazole

(Imidazole buffer substance ACS, Merck) are added to bring sample pH to ~ 8. Afterwards 1.5 g w. w. powdered cadmium is added to each centrifuge tube. Placed on a horizontal shaker the centrifuge tubes were shaken sideways with 240 rpm for > 17 hrs. Cadmium solids are separated from the water sample by centrifugation (10 minutes at 2000 rpm). Afterwards nitrite is measured in the water samples by the spectrometric method described in (21) to verify the complete reduction of nitrate to nitrate. Table 3 shows the refined experimental set up for the calibration experiment and Figure 3 illustrates the cadmium reduction step.

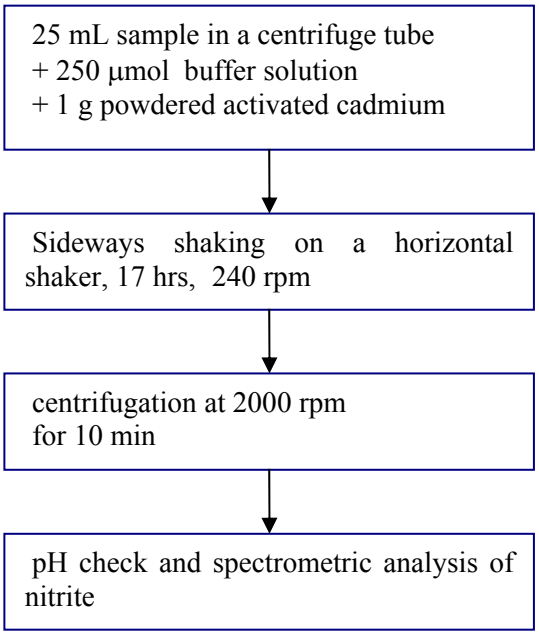

Figure 3: Reduction of nitrate with the cadmium reduction method (4)

3.3.3. Conversion of nitrite to nitrous oxide using sodium azide / acetic acid reagent

The procedure and the precautionary measures of the sample preparation for the conversion of nitrite to N_2O in water samples are described in Figure 4. Beforehand, it is recommended by (22) to place the headspace vials containing the water samples in the water bath at 30 ºC.

Table 3: Experimental set up for the calibration experiment C9

Sample Id.	C9 1a	C9 1b	C9 2a	C9 2b	C9 3a	C9 3b	C9 4a	C9 4b	C9 5a	C9 5b
Cd [g w.w]	1.1	1.0	1.1	1.1	1.2	1.2	1.2	1.1	1.1	~ 1
nitrate solution matrix solution	No 25 mL 0.5 M NaCl		0.5 mL 1mM IAEA-NO3 24.5 mL 0.5 M NaCl		0.5 mL USGS 34 24.5 mL 0.5 M NaCl		0.5 mL USGS 35 24.5 mL 0.5 M NaCl		0.5 mL Merck KNO_3 24.5 mL 0.5 M NaCl	
c NO_3^- nominal [µmol·L^{-1}]	20	20	20	20	20	20	20	20	20	20
c NO_2^- after reduction [µmol·L^{-1}]	0	0	17	17	17	17	19	18	19	19
sub-samples for conversion to N_2O	C9 1aa C9 1ab	C9 1ba C9 1bb	C9 2aa C9 2ab	C9 2ba C9 2bb	C9 3aa C9 3ab	C9 3ba C9 3bb	C9 4aa C9 4ab	C9 4ba C9 4bb	C9 5aa C9 5ab	C9 5ba C9 5bb

Procedure | **Safety instructions**

All steps

Lab protective equipment: googles; lab coat, fume hood; proper gloves; extinguisher

1. Step: 2 M NaN_3 solution (15 mL)

Weighing **1.95 g NaN_3**

↓

Dissolve **1.95 g NaN_3** in **15 mL MilliQ** in a **40 mL headspace vial**

All manipulations are done in the fume hood. weighing in sealed HS-space vials

2. Step: acetic acid/sodium azide reagent (30 mL)

Add **15 mL 20% acetic acid** to **15 mL 2 M NaN_3** within a glass vial, sealing it immediately! Mix the liquids well. **Mark the glass vials as toxic!!!**

Preparation of buffered azide needs to be in the hood due to formation of mildly volatile HN_3 at acidic pH which is both toxic and explosive (if concentrated). HN_3 causes the brown coloration. The azide reagent is kept in the hood.

3. Step: Injection of the acetic acid/sodium azide reagent to the sample

Pierce the septa of the **acetic acid/sodium azide reagent vial** with a 1 mL syringe and dray 0.8 mL of the reagent with another needle of a syringe.

Syringes containing the toxic reagent. Gloves as thick as possible!

↓

Inject the **0.8 mL of the acetic acid/sodium azide reagent vial** in a 20 mL Teflon sealed **headspace vial** containing 3 mL sample and 13 mL 0.5 M NaCl

↓

Place the vials in a **30 °C water-bath** for **60 minutes**

4. Step: Stopping the nitrite to nitrous oxide reduction

Inject **0.3 mL of 6 M NaOH** into each sample vial

↓

Samples are ready to measure in the GasBench II

NaOH is added after the reaction with azide to convert remaining reagent to N_3^- so that there are no safety problems when samples are removed from the fume hood and transferred to analysis. Reaction solution must be treated as toxic waste.

Figure 4: Reduction of nitrite to nitrous oxide – Instructions for the day of the run (3, 4, 21)

3.4. Online extraction, purification and preconcentration of nitrous oxide

The Finnigan™ Gasbench II has been modifed with the Thermo Fisher Scientific "Denitrification Kit for GasBench II" (5). The analytical system consists of the following units:

1. Autosampler: GC PAL
2. Automated sample extraction system: Finnigan™ GasBench II with the installed Denitrification Kit (modifications according to the publication by Cascotti et al. (2002))
3. CF-IRMS (DELTA V Advantage, Thermo Scientific)
4. Computer software: Isodat 2.5, Thermo

The delta ^{15}N and delta ^{18}O values of nitrate in water samples are analysed after reduction to N_2O:

1. reduction step: nitrate is reduced with cadmium to nitrite

2. reduction step to N_2O: nitrite is converted to N_2O by an acetic acid/sodium azide solution.

The last reduction step takes place in the sealed headspace vials where N_2O is partioned between the gaseous and the liquid phase.

The N_2O in the headspace vials serves as the analyte for the CF-IRMS. The N_2O is purged from the water sample into the gas phase by a purging needle and is then extracted from the headspace vials by a measuring needle. Before entering the modified GasBench II the N_2O gas sample is first separated from water vapour by a Perma Pure water trap and from water vapour and CO_2 by the $Mg(ClO_4)_2$/Ascarite trap. Afterwards the sample is introduced in the "Load mode" via the 8-port Valco valve (Figure 5) to the nickel trap (T1) which is immersed in liquid nitrogen. Thus the N_2O is condensed due to the low temperature (T= - 196 ºC). The remaining gases are escaping at the end of the nickel trap through the capillary connected one port of the 8-port Valco valve which is open in the load mode through the venting 2 m stainless capillary venting port.

This takes 800 s or the predefined extracting time and the 8-port Valco valve is now changing to the inject mode. Simultaneous the Helium is back flushing through the $Mg(ClO_4)_2$/Ascarite trap and the water trap in order to remove water vapour.

In the inject mode (Figure 6) the N_2O sample is transported with a slow Helium flow rate to the fused silica trap (T2), where it is concentrated into a smaller volume. Subsequent the gas samples passes through the Poraplot-Q gas chromatography column (T = 20 ºC) and the Nafion water trap. Finally, the N_2O sample is reaching the sample open split and is introduced to the Delta V Advantage.

Pulses of references gas are introduced to the Delta V Advantage by the reference open split. Reference peaks arriving first at the mass spectrometer and afterwards the sample peak appears.

The default values for the helium flow rates of the GasBench II are the following:

- Water trap 2 6 to 8 mL · min^{-1} at vent
- PERMA PURE 6 to 8 mL · min^{-1} at vent
- Helium vent (Valco) 8 to 12 mL · min^{-1} at vent
- Injection needle (purging) 27 to 28 mL · min^{-1} at the needle
- Injection needle (measurement) in inject mode 12 mL · min^{-1}
- 2 m stainless steel vent 5 mL · min^{-1}

The time event list how the different parts of the GasBench II are regulated by the ISODAT 2.5 software is given in Table 4.

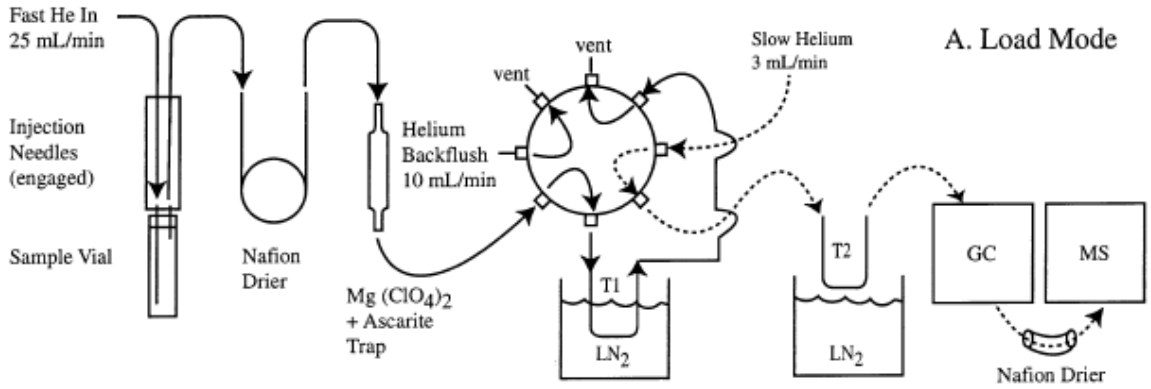

Figure 5: Finnigan™ GasBench II in load mode - on-line gas extraction, purification and pre-concentration system (5)

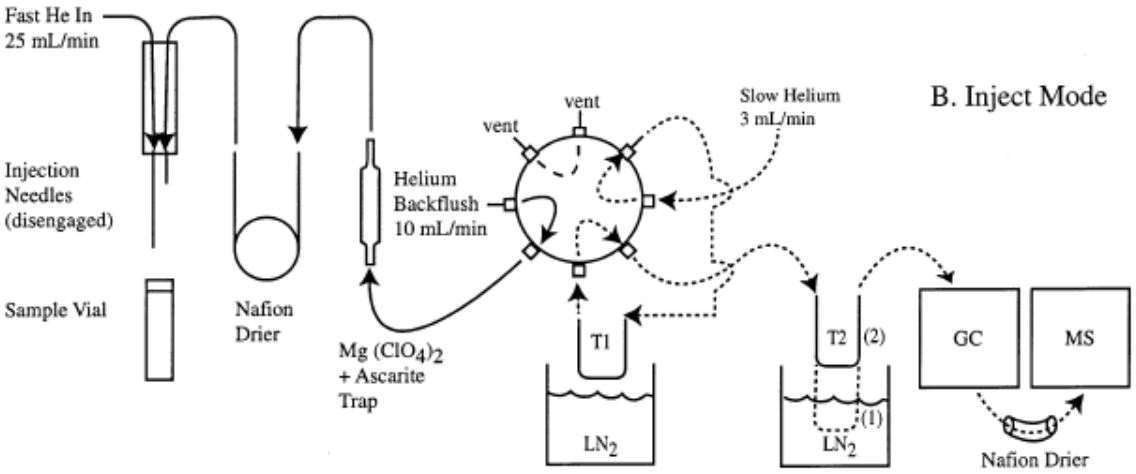

Figure 6: Finnigan™ GasBench II in inject mode - on-line gas extraction, purification and pre-concentration system (5)

Table 4: Time events list for the Finnigan™ Gasbench II

Time [s]	reference gas open split	sample open split	8-portvalve	Liquid nitrogen traps	
				trap 1	trap 2
0			load mode	in	
600 to 780	open 20 s / close 20 s				
800		open	inject mode		in
860				out	
1200					out

1180 to 1360	open 20 s / close 20 s				
1180 to 1360	open 20 s / close 20 s				
1410		close			
1650	aquisition end				

3.5. Method performance, isotope ratio standardisation and measurement uncertainty

The isotope ratio standardisation is realized as described in (3) with the isotopic reference material USGS 34 and USGS 35 and IAEA-N3 (20) in order to determine the $\delta^{18}O$ and $\delta^{15}O$ values. Recently, results of a comprehensive interlaboratory comparison were published (19) and these values are used in this study to calibrate the method. Figure 7 illustrates the standardization procedure.

The trueness and performance of the method is investigated by analysing a nitrate working standard dissolved in a 0.5 M sodium chloride solution. This working standard is measured as well by Thermal Conversion / Elemental Analyser Continuous Flow Mass Spectrometry. The method was calibrated with nitrate isotope reference materials which have been dissolved as well in a 0.5 M sodium chloride solution. The working standard and the isotope standard samples were undergone the same analytical procedure. All were analysed with the Gasbench II / Delta V advantage system and were standardized versus a N_2O reference gas resulting in δ15N and $\delta^{18}O$ values. The isotopic ratios of the sample nitrous oxide and of a pure nitrous oxide gas injection are used to calculated the $\delta^{18}O$ values (3):

Equation 1: $$\delta^{18}O_{N2O} = \frac{R_{sample} - R_{std}}{R_{std}} \cdot 1000$$

$\delta^{18}O_{N2O}$ relative difference of isotope ratios vs. nitrous oxide reference gas

R_{sample} ratio of the masses 46/44 in the sample

R_{std} reference gas nitrous oxide

In order to report the $\delta^{15}N$ and $\delta^{18}O$ values to international calibrated scale a linear function of the published δ values for nitrate reference materials against its measurements standardized to the reference gas was used (Equation 2). Thus, this linear function was introduced to calculate the measurement result and the measurement uncertainty according to (24) in Gum Workbench (25). The $\delta^{18}O$ results of the measurements of the isotope standards and the working standards which were calculated by ISODAT 2.5 were imported into Gum Workbench 2.3 as observations (Type A uncertainty, normal distribution). Whereas the $\delta^{18}O$ values of the isotope standards (19) and the working standard (section 3.2) are entered as Type B uncertainties (normal distribution) into Gum Workbench.

Equation 2: $$\delta^{18}O_{nitrate} = \frac{\delta^{18}O_{N2O-}b_{std}}{m_{std}}$$

$\delta^{18}O_{nitrate}$ relative difference of isotope ratios vs. VSMOW

$\delta^{18}O_{N2O}$ relative difference of isotope ratios vs. reference gas

b_{std} y intercept of the curve made by nitrate reference materials measured vs. reference gas of known $\delta^{18}O_{nitrate}$ reported to VSMOW

m_{std} slope of the curve made by nitrate reference materials measured vs. reference gas of known $\delta^{18}O_{nitrate}$ reported to VSMOW

The $\delta^{18}O$ values of the working standard measured with the TC/EA (section 3.2) and measured with the GasBench II are assumed to be equal if the following equation is fulfilled:

Equation 3: $$\varepsilon_1 = \left|\delta^{18}O_{WS\_TCEA} - \delta^{18}O_{WS\_GasBench}\right|$$

Equation 4: $$\varepsilon_1 \leq k \cdot u(\delta^{18}O_{WS\_TCEA} - \delta^{18}O_{WS\_GasBench})$$

ε_1	difference
$u(\delta^{18}O_{WS\_TCEA} - \delta^{18}O_{WS\_GasBench})$	uncertainty of the measured difference
k	coverage factor (k = 2)
$\delta^{18}O_{WS\ GasBench\ vs.\ VSMOW}$	$\delta^{18}O$ measured with the GasBench II vs. VSMOW
$\delta^{18}O_{WS\ GasBench\ vs.\ VSMOW}$	$\delta^{18}O$ measured with the TC/EA vs. VSMOW

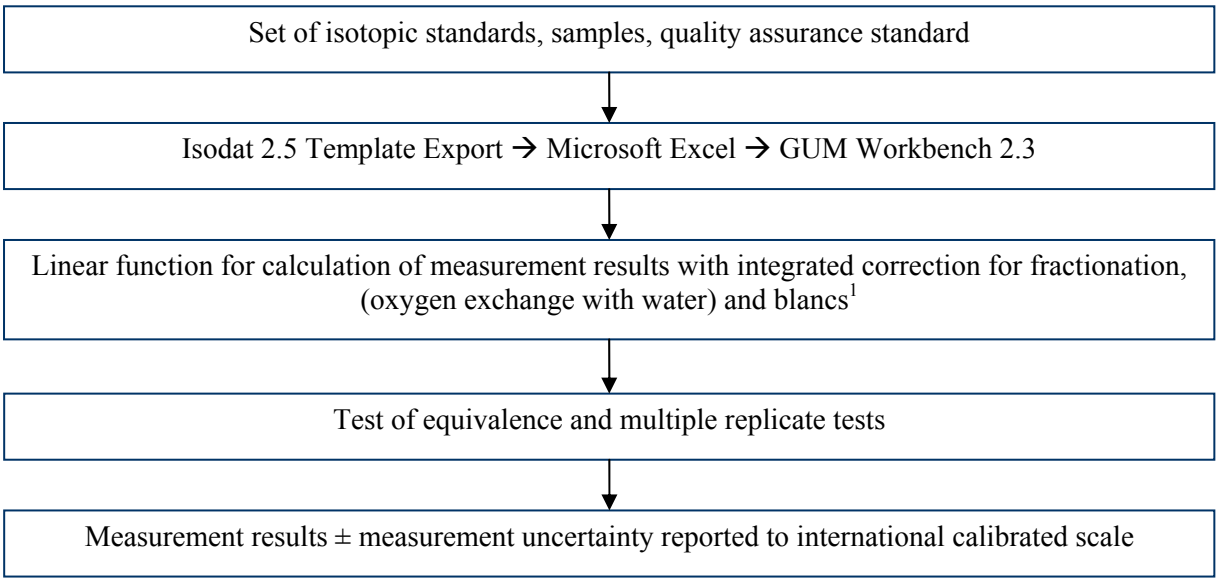

Figure 7: Isotope ratio standardisation and measurement uncertainty

4. RESULTS AND DISCUSSION

4.1. Optimization of the CF-IRMS measurements

There are still some improvements of the method possible to achieve better reproducibility. The Delta V advantage is very sensitive to a background of water which has been always introduced by the purging of the water samples into the system. High water backgrounds affected the reproducibility of the measurements. In order to reduce the background of water it is required to heat the analyser heater of the ion source for 48 hours once a week, preferably over the weekend and as well the GC column overnight at 120 ºC. The measurement sequence ends always in inject mode, thus between some measurements there will be an Helium back flush through the Magnesium perchlorate Ascarite trap and through the Nafion water trap in order to remove the water.

To identify any unreproducibility of the system injections of the reference gas has been integrated in the time event list (Table 4).

Other solutions to reduce the water background sustainable would be the installation of a third Nafion water trap or the trapping of the water vapour with a dry ice trap. Another possibility is to connect the GasBench II to a different Mass spectrometer which is not so sensitive for water.

4.2. Optimization of the sample preparation

In the method of (3, 4, 21) an imidazole buffer is used to buffer the system. After the reduction of nitrate to nitrite, with this buffer the pH has been usually between 10 and 11.

The use of Magnesium chloride as a kind of buffer has been tested. Results show that 250 µl of 1 M solution achieve a pH ~ 9 in the batches.

$$Mg^{2+}\,(aq) + 2\,OH^{-}\,(aq) \rightarrow Mg(OH)_2\,(s)$$

Results show, that in a 0.5 M sodium chloride blank which passes all reduction steps N_2O can be measured in the headspace vials. Whereas nitrite is not detectable in the same 0.5 M sodium chloride blank sample after passing the cadmium reduction step which means that neither nitrate nor nitrite can be introduced via the MilliQ or the chemicals used for the cadmium reduction step. N_2O blank is still not fully under control and this has to be investigated further. Since the correction is included in the calibration it does not affect the result assuming that the blank effect is the same in the isotopic standard and in the samples.

4.3. Optimization of the sample run time

The GasBench II has been installed with a flow rate of 11 $mL \cdot min^{-1}$ at the injection needle (purging). In the installation manual of the denitrification kit a flow rate between 12 and 18 $mL \cdot min^{-1}$ is recommended whereas (3) propose a flow rate of 30 $mL \cdot min^{-1}$. First it has been adjusted by shortening the capillary of the Helium supply for the inject needle (purging) to a flow rate of 18 $mL \cdot min^{-1}$. But the sufficient autosampler run time for this setting was still 1600 seconds. After increasing the flow rate to 28 $mL \cdot min^{-1}$ a purging time / autosampler runtime of 800 seconds has been sufficient as shown by the results in Table 5. The $\delta^{18}O$ and $\delta^{15}N$ values which were achieved with the different purging times of 800, 1600 and 3200 seconds are comparable meaning that the deviations between the values are within the range of standards deviations of the measurements.

Table 5: Results of a 20 µmol·L^{-1} potassium nitrate artificial sample measured with different purging times

purging time	mean[1]		standard deviation[1]	
[s]	$\delta^{18}O_{gas} \cdot 10^3$	$\delta^{15}N_{gas} \cdot 10^3$	$\delta^{18}O_{gas} \cdot 10^3$	$\delta^{15}N_{gas} \cdot 10^3$
800	10.75	10.19	0.14	0.09
1600	10.65	10.65	0.68	0.65
3600	11.73	10.10	0.36	0.02
all	11.04	10.32	0.59	0.30

[1] $\delta^{18}O$ and $\delta^{15}N$ are measured versus the reference gas

4.4. Relative isotope amount ratio analysis of nitrate in aqueous samples

The result of the nitrate working standard for the $\delta^{18}O$ measured with the GasBench II/ Delta V Advantage was 24.5 ‰ vs. VSMOW and the expanded measurement uncertainty[1] (k = 2) 1.3 ‰ vs. VSMOW (Table 6). The coefficient of determination of the underlying linear function for the determination of relative difference of isotope-amount ratios of O is $R^2 = 0.9947$.

Whereas the measurement result ± expanded measurement uncertainty was 23.3 ± 0.64 ‰ vs. VSMOW with TC/EA CF-IRMS. The difference of 1.2 ‰ is smaller than the expanded measurement uncertainty of this difference meaning the test of equivalence - the criterion of Equation 4 - has been fulfilled (

Table 7). The measurement uncertainty of the TC/EA CF- IRMS is smaller because only the solid nitrate was analysed and no other sample preparation steps contribute to the uncertainty.

Usually, in the literature experimental standard deviations of replicate analysis are given to evaluate the performance of the method. Table 1 gives an overview for the published values for the analytical precision. In this exploratory an analytical precision of 0.23 to 0.63 ‰ has been achieved for the $\delta^{18}O$ and 0.1 to 0.2 ‰ for $\delta^{15}N$ analysis (standard deviations of duplicate samples). Thus, the analytical precision is comparable or better to the other published data in Table 1.

Until the end of the exploratory research project it has not been possible to achieve acceptable calibrated values for the relative N isotope-amount ratios of the nitrate working standard. Therefore further optimizations of the method are required (see also section 4.1 and 4.2) and further investigation on the trueness, repeatability and reproducibility are necessary including the analysis of sea water samples and other aqueous samples as well for the analysis of $\delta^{18}O$ values.

[1] $U = k \cdot u$ with U: expanded measurement uncertainty, k: coverage factor, u: combined standard uncertainty

Table 6: Measurement result and measurement uncertainty of an duplicate analysis of the working standard (WS, artificial sample containing 20 µmol·L^{-1} potassium nitrate (Merck) and 0.5 mol·L^{-1} sodium chloride)

quantity	value (‰)	standard uncertainty (‰)	uncertainty contribution
$\delta^{18}O_{WS\ GasBench\ vs.gas}$	10.579	0.117	35.3 %
$\delta^{18}O_{USGS\ 35\ vs.\ VSMOW}$	56.81	0.31	3.7 %
$\delta^{18}O_{USGS35\ vs.\ gas}$	21.047	0.329	45.2 %
$\delta^{18}O_{USGS34vs.\ VSMOW}$	-27.78	0.37	2.1 %
$\delta^{18}O_{USGS34\ vs.\ gas}$	-5.025	0.168	4.7 %
$\delta^{18}O_{IAEA\ vs.\ VSMOW}$	25.32	0.29	2.5 %
$\delta^{18}O_{IAEA\ vs.\ gas}$	9.872	0.145	6.6 %
$\delta^{18}O_{WS\ GasBench\ vs.\ VSMOW}$	**24.496**	**0.64**	

Table 7: Test of equivalence for the measurement results (Table 6) of duplicate analysis of a working standard (artificial sample containing 20 µmol·L^{-1} potassium nitrate (Merck) and 0.5 mol·L^{-1} sodium chloride) compared to measurements of $\delta^{18}O$ of the same potassium nitrate (Merck) with the TC/EA.

quantity	value (‰)	standard uncertainty (‰)	uncertainty contribution
$\delta^{18}O_{WS\ Gas\ Bench\ vs.\ gas}$	10.579 ‰	0.117	28.5 %
$\delta^{18}O_{USGS\ 35\ vs.\ VSMOW}$	56.81	0.31	3.0 %
$\delta^{18}O_{USGS\ 35\ vs.\ gas}$	21.047	0.329	36.4 %
$\delta^{18}O_{USGS\ 34\ vs.\ VSMOW}$	-27.78	0.37	1.7 %
$\delta^{18}O_{USGS\ 34\ vs.\ gas}$	-5.025	0.168	3.8 %
$\delta^{18}O_{IAEA\ vs.\ VSMOW}$	25.32	0.29	2.0 %
$\delta^{18}O_{IAEA\ vs.\ gas}$	9.872	0.145	5.3 %
$\delta^{18}O_{WS\ TC/EA\ vs.\ VSMOW.}$	23.3	0.315	19.4 %
ε_1	**1.196**	**0.716**	

5. CONCLUSION

McIlvin and Altabet (3) published a sample preparation method ("cadmium reduction method" or "azide method") which converts NO_3^- to N_2O by a two step chemical reduction procedure; subsequent, N_2O is analyzed for the $\delta\ ^{15}N$ and $\delta^{18}O$ in the same way as described for the denitrifier method (5, 17). The following characteristics of the cadmium reduction method can be assessed as advantageous 1. less laboratory intensive, results can be available within one day, 2. less consumables, cadmium can also be reused, 3. concentrations of 0.5 L^{-1} µmol can be analysed also in small sample volumes, 4. no interferences with high halide concentrations and toxic substances, 5. prospective for automatisation with a high sample throughput. The following issues can be marked as disadvantageous: 1. method requires environmental and health protection measures due to the toxicity of the cadmium and sodium azide, and to the risk that under acidic conditions HN_3 is formed which is extremely toxic, volatile and if concentrated also explosive, 2. reaction conditions must be controlled properly otherwise an exchange of oxygen with water can take place, 3. results needed to be adjusted for blanks, oxygen exchange and fractionation. The method of McIlvin and Altabet (4) is intricate (22), thus, it requires training and comprehensive experience.

The cadmium reduction method has been shown to work for sea water samples (3, 13, 26) which previously could only be measured with the 'bacterial denitrification method' (5, 17). As the 'cadmium reduction method' is still limitedly applied for $\delta^{15}N$ and $\delta^{18}O$ measurement in a few source apportionment studies in groundwater (27), rivers (28), coastal waters (13, 26). It needs to be further consummated in future in order to provide a validated fast and robust method for source apportionment studies.

The results of this exploratory show that N_2O is a useful analyte for the analysis of the isotopic composition of for the relative isotope amount ratio of O and N in nitrate. Nitrous oxide can be easily separated by purging from the water sample with the GasBench II on-line gas extraction, purification and pre-concentration system (5). With a method run time of 1650 s per sample it is possible to run 48 samples per day including a set of isotope standards, a blank sample and a quality assurance sample.

The 'cadmium reduction method' is very useful for seawater and aqueous samples with high concentrations of anions because the anions speed up the reduction (3). A final evaluation of the method requires testing the performance of the method for nitrate isotope analysis on real seawater samples, nitrate source samples (sewage, industrial wastewater, animal manure) and fresh water samples. For measurements of seawater samples the isotope reference materials and working standards have to be matrix adapted by dissolving them into zero nutrient seawater.

A quantification of the fractionation, oxygen exchange with water and blanks effects is still required unless a correction for is included in the calculation of the measurement results. The minimizing of fractionation, oxygen exchange and blank effects would enable further optimization of the method.

An interlaboratory comparison with the method is planned in cooperation with the University Ghent to evaluate the method for surface water samples in comparison with the anion exchange method and with the bacterial method.

With the adoption of the Marine Strategy Framework Directive (2) the member states are requested to develop programmes of measures towards good environmental status by 2021. Nitrate and other nutrients are responsible for the eutrophication which affects the environmental ecological status of marine waters seriously. Studies on source apportionment of nitrate with stable isotopes in seawater are limited. A comprehensive investigation on the source apportionment of nitrate in seawater would be required to prove whether this approach is also applicable in sea water. Within the marine framework strategy the member states need also to describe and assess the environmental status of and the human impact on their marine waters. Thus $\delta^{18}O$ and $\delta^{15}N$ can be powerful indicators for the human impact on the marine environment which can be affected by the human discharges and emission of nitrate and other nutrients.

Furthermore, member states need to provide evidence that the measures which they will be taken to achieve a good environmental status by 2021 are effective. Therefore the 'cadmium reduction' method can also support the member states by studying natural attenuation processes of nitrate in sea water. Further investigations on natural

attenuation processes and as well on the nitrogen cycle with stable isotopes are necessary to have robust indicators whether measures which are taken by the member states are satisfactory to achieve a good environmental status by 2021. The 'cadmium reduction' method can easily be combined with the stable isotopes analysis of the other compounds of the nitrogen cycle: (a) nitrite by bypassing the cadmium reduction step and with $\delta^{15}N$ analysis of (b) organic nitrogen which can be determined with a prefixed persulfate oxidation (29) or (c) Ammonia by oxidation with hypobromite to nitrate which is then reduced to N_2O (30).

Alternatives for the conversion of nitrate to N_2O could be considered: This also needs to be proved in order to replace the use of carcinogenic substances as required by (31). One alternative sample preparation method for the dual isotope analysis of nitrate can be enzymatic nitrate reduction. The production of any enzyme using the recombinant DNA technology is possible by now and the biochemistry of enzymes is well understood (32). Thus enzymes of analytical grade can be used in analytical chemistry. The reduction of nitrate to N_2O requires nitrate reductase (NaR), nitrite reductase (NiR), nitric oxide reductase (NoR), coenzymes (NADH) respectively an electron donor (e.g. dyes, electrical current) and a buffer solution. An advantage of enzymatic reduction is the high specific use of the substrates. Nitrate reductase is also suitable for difficult matrices like sea water, wastewater or aqueous soil extracts (33). The proposed enzymatic reduction of nitrate can be easily integrated into online gas bench applications either by a fully automated enzyme dosing system (dosing technique like Carbonate Reaction Kit) or by the means of immobilization of the enzymes on a surface (= catalyst, similar to D/H-analysis by Platinum catalyst with the GasBench II). Immobilization techniques for NaR and NiR are described in the literature (34, 35).

Another approach could be the reduction of nitrate with catalysts which could be similar applied as D/H-analysis by Pt-catalyst with the GasBench II. The reduction of nitrate to nitrogen by catalyst is investigated for wastewater treatment technologies (36). One product which develops during this reduction is N_2O and depending on the kind of catalyst and the reaction conditions the amount of N_2O differs (37, 38). Especially, a sample preparation with catalysts would be a challenging approach because there is the potential for a complete automated sample preparation.

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EUROPEAN COMMISSION
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Detection of potentially allergenic peptides derived from bovine milk enzymatic hydrolysates in food matrices by a proteomic approach.

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SUMMARY

The increasing popularity of fitness and health care is a driving force for the food industry to develop new food products that can fulfil the wishes of a health conscious population. The use of food or protein processing methods as well as the inclusion of peptides brings new functionalities to food products. Milk proteins contain such bioactive peptides that can be liberated by enzymatic hydrolysis. Milk-derived peptides with health benefits are likely to be used more and more in food products. However, the use of milk-derived peptides as ingredients has to be indicated on the label to comply with European legislation. Since immunochemical techniques were shown in our previous exploratory research (2007) to hardly detect milk-derived peptides, other techniques are required. Capillary electrophoresis-mass spectrometry (CE-MS) is a technique that might be used to detect and identify milk-derived peptides in food. For this purpose a method based on CE-MS was developed for the detection of milk-derived peptides that were spiked into fruit juices. An extraction method based on solid phase extraction was developed to extract and concentrate milk-derived peptides before their analysis by CE-MS. Milk-derived peptides obtained after enzymatic hydrolysis were spiked at different levels in orange juice or multifruit juice. Peptides derived from whey proteins and caseins could be detected and identified in both type of juices.

ABBREVIATIONS: Caseins (CN); Sodium caseinate (Na-CN); Whey protein concentrate (WPC); Beta-lactoglobulin (β-LG); Solid Phase extraction (SPE); Capillary electrophoresis- Mass spectrometry (CE-MS).

INTRODUCTION

The increasing interest in health and well being promoted by food industries is associated with the development of new innovative food products that claim to have a positive impact on body functions and ultimately on health [1]. To manufacture such nutraceutical food products and highlight the desired functional properties, industries implement sophisticated processes that affect proteins responsible for the properties of the food [2]; [3].

Food industries increasingly utilize protein hydrolysis as a manufacturing process to make use of the physical and functional properties of the emerging peptides, that are often more potent than the proteins themselves [4]; [5].

Milk proteins are known to be an extremely rich source of encrypted bioactive peptides possessing multiple potent beneficial health effects like antimicrobial, antioxidative, antithrombotic, antihypertensive or immunomodulatory activities [6]; [7]. Therefore milk-derived peptides are good candidates for their use in the formulation of food products with diverse properties [2].

However, milk proteins are also famous for triggering food allergic reactions in children as well as in adults who did not outgrow their milk allergy [8]; [9]. Hypoallergenic formulas based on peptides derived from milk enzymatic hydrolysis are a substitute diet for the allergic population but several adverse reactions to those formulas have been reported in allergic children [10]; [11]. Processed foods such as cheese have also been shown to induce allergic reactions in allergic patients [12]; [13] due to the presence of residual allergic epitopes of the proteins [14]; [15]. Therefore, even after degradation of the offending proteins, there is a potential allergenicity of the emerging peptides. Since peptides with a length of nine amino acids are already able to trigger an allergic reaction in sensitised patients, there is a substantial health risk for the allergic consumer when exposed to those new products supplemented with milk peptides. To assure safety with regard to allergic consumers, the detection of milk-derived peptides used in food products is necessary. European legislation [16] specifies that the use of milk or its derivatives as an ingredient has to be declared on the label of products containing it. To support this legislation, analytical methods are available to detect milk proteins in food matrices (e.g. ELISA, electrophoresis, LC-MS). However, there is no method specific for peptides except immunodetection technique [17]. The ER2007 project we carried out investigated the relevance of using the standard techniques like ELISA and Western blotting for the detection of potentially allergenic milk hydrolysates. It has demonstrated that these immunodetection techniques were not suitable to detect milk-derived peptides [18]. Those immunodetection techniques designed for the recognition of intact proteins fail to detect milk-derived peptides in hydrolysates especially after ultrafiltration. Despite a drastic decrease of the detectable amount of proteins, associated with a loss of antigenicity (disappearance of epitopes) of hydrolysed milk protein formulas, there is a persistence of peptides that can hold epitopes (mainly linear), which are still able to induce immune reaction in allergic patients [19]. Therefore there is a clear need to develop alternative methods that are able to detect allergen-derived peptides and not only the offending proteins. The same exploratory research project of 2007 has suggested that a capillary electrophoresis-mass spectrometry (CE-MS) method could be a suitable confirmatory technique for the detection and the identification of milk-derived peptides

[18]. This technique allows indeed an unambiguous identification of peptides present in the hydrolysates.
To address the presence of protein derived peptides in food products, it was necessary to analyse a controlled test material, i.e a sample matrix that should contain a definite amount of peptide hydrolysates. To get enough material to work with, new batches of milk protein hydrolysates (e.g. from Na caseinate and whey protein) were produced at larger scale with food grade enzymes from *Bacillus amyloliquefaciens, Aspergillus oryzae*, trypsin, pancreatin, used in the food industry as carried out during the 2007 exploratory research project [18]. After characterisation of the new batch of hydrolysates in term of protein concentration and degree of hydrolysis, the different hydrolysates were incorporated into a food matrix and more specifically commercially available juices. The peptide hydrolysates spiked in different amounts into two types of fruit juices were extracted with a newly set up solid phase extraction procedure. Finally the presence of milk-derived peptides in juices was investigated with the new capillary electrophoresis mass spectrometry method.

1. MATERIAL AND METHODS

1.1. Sample preparation

1.1.1. Production and characteristics of batches of milk hydrolysates

1.1.1.1. Milk protein hydrolysis

Industrially produced sodium caseinate (Na-CN; 90% protein) and whey protein concentrate (WPC; 75% protein) were hydrolysed in the same manner as described in the report of the 2007 exploratory research project but at a larger scale (1 liter) [18]. Briefly, milk powders (Na-CN, WPC) were dissolved in distilled water to obtain 5 % (w/v) solutions (i.e 50 g in 1 L distilled water). WPC solutions preheated for 15 minutes at 90 °C before hydrolysis, and Na-CN solutions were enzymatically hydrolysed at 50 °C with 1% (w/w) trypsin, pancreatin or a combination of 2 proteases from *Bacillus amyloliquefaciens* and from *Aspergillus oryzae* respectively (Sigma Aldrich; St Louis, MO, USA) at their optimal pH controlled with a Titrino pH system (Methrom AG, Herisau, Switzerland) [18].

Hydrolysate aliquots of 200 mL were collected at 5, 30, 60, 180 and 360 minutes respectively, and were immediately heat inactivated for 15 minutes at 90 °C. The resulting material was subsequently centrifuged for 30 minutes at 3000 x g at room temperature. The supernatant containing the peptides was harvested and stored at -20 °C before its analysis. Hydrolysate samples obtained were coded with a letter corresponding to the protein source (C or W for CN or WPC) followed by a second letter referring to the protease used (T, A/O, P for Trypsin, the combination of proteases from *Amyloliquefaciens* and *Oryzae* and pancreatin respectively) and finally a number corresponding to the hydrolysis time (5, 30… 360 indicating the minutes of hydrolysis).

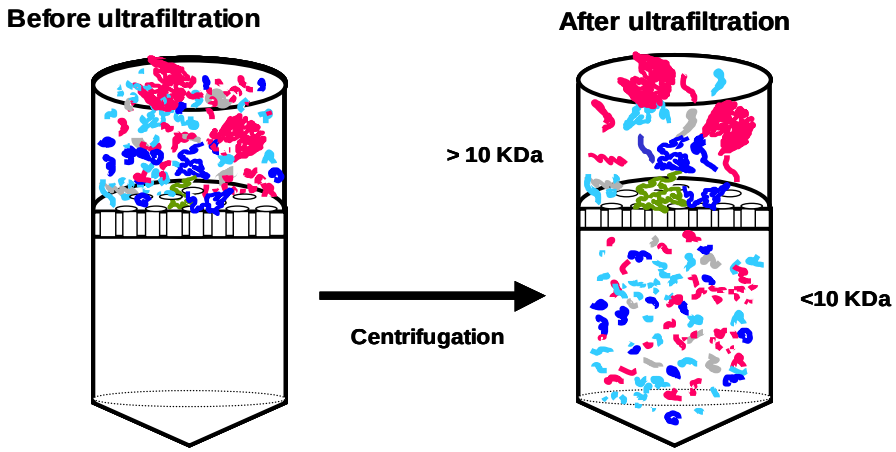
1.1.1.2. Ultrafiltration

To remove poorly hydrolysed proteins, the hydrolysates were submitted to ultrafiltration in ultracentifugal devices possessing 10 KDa membranes (vivacell 100; Sartorius Stedim

Biotech, Goettingen, Germany) at 2000 xg. The principle of ultrafiltration is depicted in **Figure 1**.

The peptides below 10 KDa are able to pass through the membrane into the lower part of the centrifugal device. The resulting ultrafiltrated hydrolysates (permeates) were collected and were used to spike juices prior to CE-MS analysis.

Figure 1: Ultrafiltration principle



1.1.1.3. Hydrolysate characterisation

1.1.1.3.1. Protein quantification based on nitrogen content

The protein concentration of the milk hydrolysates before and after ultrafiltration was estimated from the measurement of the total nitrogen content by the Dumas method. The quantity of nitrogen converted into protein equivalent was determined with the Elementar Vario MAX CN analyser (Elementar GmbH, Hanau, Germany) using aliquots of 250 mg hydrolysate and 200 mg of calibrant (glutamic acid) supplied by VWR International (West Chester, PA, USA).

1.1.1.3.2. Degree of Hydrolysis

The degree of hydrolysis was assessed on the total hydrolysate with the TNBS (trinitro benzene sulfonic acid) assay described in the 2007 exploratory research project [18]. Diluted hydrolysate samples (15 µL) and L-Leucine solutions (standard) in SDS (1 % w/v) were incubated into a 96 well plate at 50 °C for 60 minutes in a thermostated chamber with TNBS reagent (45 µL) in alkaline buffer (45 µL 0.2125 M sodium phosphate buffer pH 8.2). After acidification of the wells with 0.1 N HCl (90 µL), the absorbance values were measured at 340 nm using a multilabel plate reader (Victor, Perkin Elmer, Waltham, MA, USA).

1.2. Spiking of the matrices

1.2.1. Types of matrices

The detection of milk-derived peptides spiked into food matrices was performed using commercially available juices. For this purpose, two types of juices: orange juice (Minute maid) and Multi-vitamin fruit juice from Delhaize (composed of 12 fruits: orange, pineapple, apple, grape, passion fruit, pear, lemon, banana, nectarine, mango, guava, papaya) respectively were tested (**Annex 1**).

1.2.2. Method of spiking

Different spiking levels in juices respectively 0.01%, 0.05%, 0.1%, 0.5% (v/v) were established. The corresponding spiking levels were obtained by preparing a starting solution of juice spiked with 1% hydrolysate (v/v), which was serially diluted with a blank juice as shown in **Figure 2**.

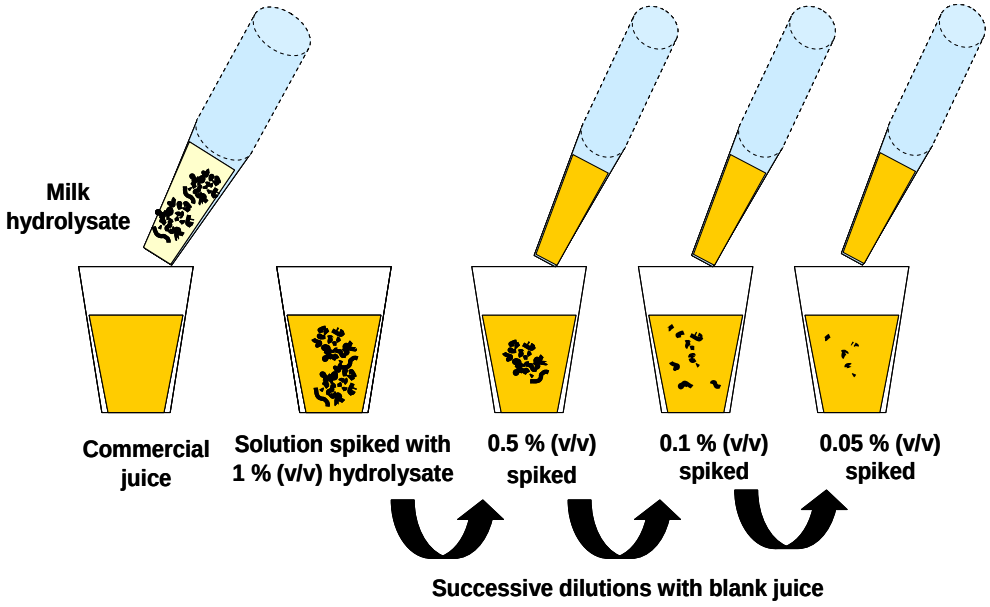


Figure 2: Spiking mode.

The spiked juices were centrifuged 10 minutes at 10000 rpm (Eppendorf, rotor 31346) in order to precipitate and hence eliminate insoluble particles. The supernatants were collected and submitted to the extraction procedure.

1.2.3. Peptide extraction by solid phase extraction

For peptides, two approaches can be considered for their purification: the use of either ion exchange solid phase by modulating the charge state of the peptides or the reversed phase solid phase system in which the hydrophobicity of the peptides is the main factor of retention. A new type of SPE adsorbents by its bifunctional extraction capacity offers the possibility to combine ion exchange and hydrophobic interactions. The SPE cartridge (Oasis MCX) employed for the extraction consisted of a Mixed-mode Cation-eXchange and reversed-phase sorbent highly selective for basic compounds (Waters, Milford, MA, USA).

1.2.3.1.1. Peptide extraction with SPE cartridges

Before analysing food matrices (fruit juices) it was necessary to test the feasibility of the SPE system for peptide extraction. For this purpose milk-derived peptides from the ultrafiltrated hydrolysates produced in 2007 were directly extracted with SPE MCX devices. After the conditioning of the SPE cartridges, 2 mL of each of the 12 hydrolysates (0.5% in acidified water) obtained after 6 hours of hydrolysis (CT360, CP360, CA360, CO360, CB360, CAO360 and WT360, WP360, WA360, WO360, WB360, WAO360) were adsorbed, cleaned and eluted from the SPE cartridge according to the method described in **Table 1**. The eluates, reconstituted in 120 µL of ACN/Water (50/50), 1% Formic acid, were analysed by CE-MS.

1.2.3.1.2. Extraction Methods

The extraction procedure used to extract milk-derived peptides from the juices is summarised in **Table 1**. The extraction and CE-MS method was developed based on the study of milk protein hydrolysed with trypsin. The analytes submitted to cationic exchange extraction are positively charged in low pH environment. The positively charged peptides in food matrix extracts adsorb to the preconditioned stationary phase due to electrostatic interactions between the positive charge of the peptides and the negative functional groups grafted on the stationary phase (cartridge) (**Figure 3**). After washing the stationary phase, the peptides attached to the solid phase are eluted with an appropriate solvent.

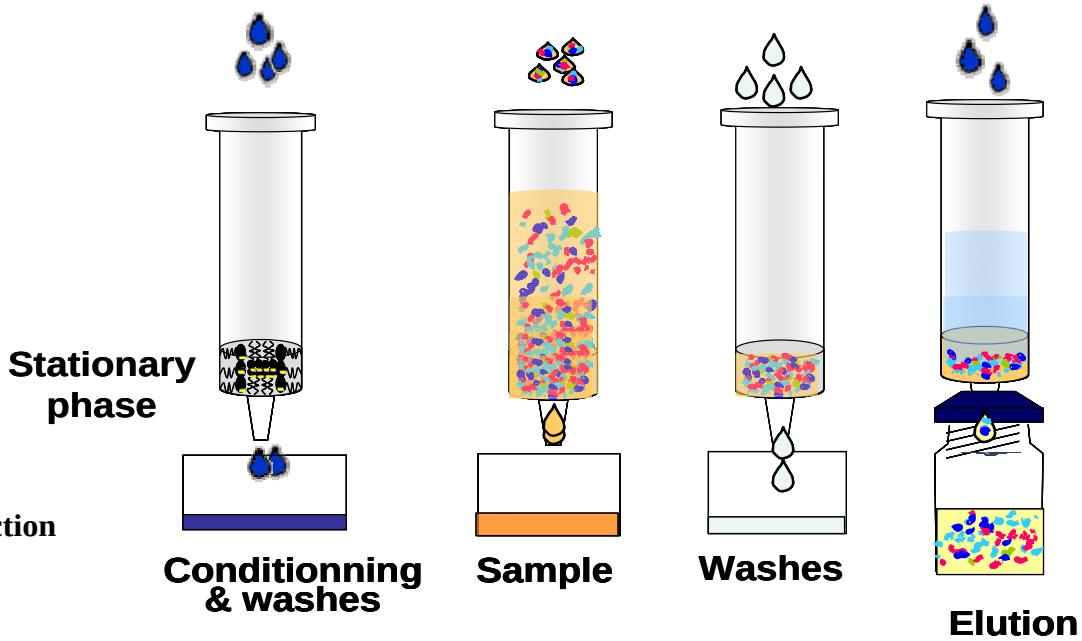


Figure 3: Extraction

Table 1: Extraction protocol with SPE devices.

Process steps	**Extraction cartridge**
	MCX
Conditioning	3 mL CH_3OH 3 mL H_2O

Loading	4 mL extract final pH around 4
Washing	2 mL CH_3OH
Eluting	3 mL CH_3OH/25% NH_4OH (95/5, v/v)
Evaporation	under a nitrogen flow
Resuspension	120 µL of acetonitrile/water (50/50) + 1%FA
Extract aspect	Transparent

The use of MCX type cartridges required that the sample was supplemented with formic acid (50 µL FA (10%) for 4 mL sample) to obtain a pH around 4 before loading on the extraction cartridge.

Milk-derived peptides extracted from juices were evaporated under nitrogen before being resuspended in 120 µL of acetonitrile/water (50/50), formic acid 1% and diluted in a buffer compatible for CE and mass spectrometry.

1.3. Analysis of peptides extracted from beverages by CE-MS

1.3.1. CE-MS method

A CE-MS method was set up for the detection and sequence identification of milk-derived peptides spiked in beverages.

Peptides present in the extracted samples were separated online by capillary electrophoresis on a P/ACE™ MDQ CE instrument (Beckman-Coulter, Fullerton, CA, USA) controlled by the Beckman 32 Karat™ Software and analyzed by electrospray ion trap mass spectrometry using a Finnigan LCQ Deca mass spectrometer (Finnigan, San Jose, CA, USA) using a coaxial capillary arrangement (**Figure 4**).

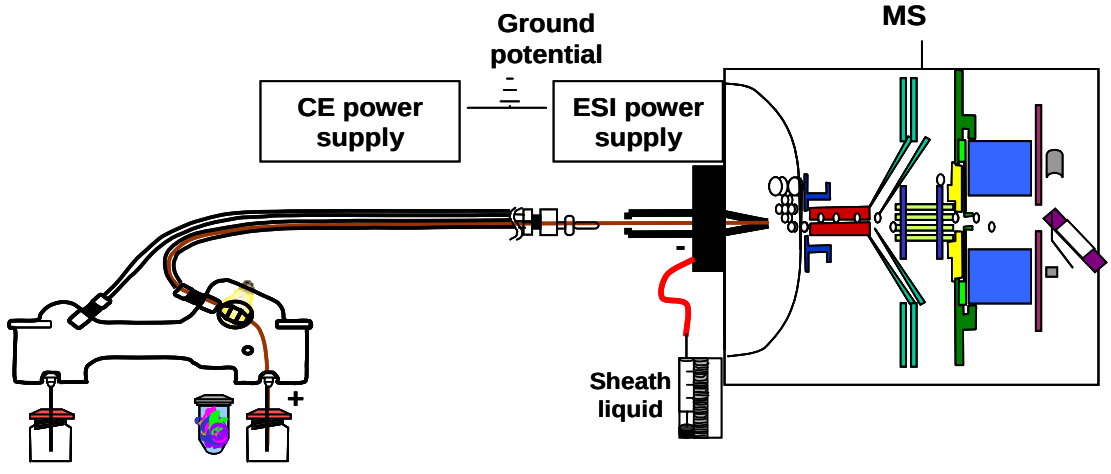


Figure 4: Schematic principle of CE-MS.

New capillaries installed for the separation of peptides needed to be initially preconditioned with a 20 minutes rinse with NaOH (1 M) followed by 10 min rinses with water and separation buffer (100 mM ammonium formate/formic acid pH 2.5). Washes of the

capillary with successively 1 M NaOH and deionised water (Millipore, Bedford, MA, USA) for 5 minutes and with the separation buffer for 8 minutes were executed between each analysis run of extracted samples.

The 2-fold diluted extracted peptide mixture was hydrodynamically injected (2 psi, 8s) into a 86-cm-long, i.d. 50 µm Xo.d. 363 µm, bare fused-silica capillary (Polymicro Technologies, Phoenix, AZ). The peptide migration was achieved in a 100 mM ammonium formate/formic acid pH 2.5 running buffer by applying and maintaining for 40 minutes a negative voltage (typically -30 kV) at the injector end of the capillary. The other end of the capillary was directed through a stainless-steel ESI probe to electrospray the eluting peptides into the mass spectrometer. Concomitantly a syringe pump delivered a coaxial sheath-liquid (0.1% formic acid in 50/50 water/methanol) at a flow rate of 3 µl/min. The ions entering the analyser were separated according to their mass to charge ratio (*m/z*) and submitted to fragmentation under a collision energy of 35%. All MS analyses (acquisition of the mass spectra and fragmentation pattern) were operated in the positive ion mode of detection. Experiments were performed scanning an *m*/z range of 350-2000 for the MS and an *m*/z range of 100-2000 for the MS/MS. The mass spectrometer was operated under data-dependent mode to automatically switch between MS, charge and MS/MS modes. Analysis of the mass spectra (MS/MS) was performed with Mascot distiller software to establish the peak list before searching the peptide sequences against the Swissprot database. Sequence searches were performed for individual charge states +1, +2, and +3.

1.3.2. Repeatability

The repeatability of the CE-MS method was verified by comparing mass spectra acquired for an extracted sample run in triplicate and also for a sample submitted to two extractions.

1.3.3. Sensitivity

Extracts of juices spiked with decreasing levels of milk-derived peptides (hydrolysates), 0.5 %, 0.1 %, 0.05 % and 0.01 % (v/v) respectively, were analysed to estimate the sensitivity of the CE-MS method.

1.3.4. Matrix effects

Two types of fruit juices were tested to determine the capacity of the technique in detecting and identifying the presence of milk-derived peptides. After analysis in orange juice, the CE-MS technique was confronted with a more complex commercial matrix, a multi vitamin juice as described in paragraph "1.2.1 Types of matrices" (**Annex 1**).

2. RESULTS AND DISCUSSION

2.1. Production and characterisation of milk hydrolysates

2.1.1. Milk hydrolysates

Six hydrolysates were produced, three obtained by hydrolysis of Na-CN submitted to digestion with trypsin, pancreatin or the combination of the two enzymes from *Bacillus amyloliquefaciens* and from *Aspergillus oryzae* and three hydrolysates derived from WPC treated with the same enzymes. Aliquots were collected during hydrolysis (5, 30, 60, 180, 360 minutes) but the analysis focused on samples obtained after the longest hydrolysis time. The hydrolysates were submitted to ultrafiltration (cut off of 10 kDa) before spiking the resulting permeates into beverages.

2.1.2. Milk hydrolysate characteristics

The protein content of the hydrolysates estimated from the nitrogen level determined with the Dumas method before ultrafiltration and in the permeates are presented in **Table 2**. The degree of hydrolysis determined in the total hydrolysates and the participation of the enzymes (proteins: therefore source of nitrogen), in the determination of the protein content are also reported in **Table 2**.

The contribution of the enzymes to the total protein content determined before ultrafiltration (0.75 up to 6% depending on the hydrolysates) was only minor. Thus, the involvement of the enzymes in the protein concentration of the ultrafiltrated hydrolysates should be relatively small despite the partial degradation of the enzymes during their heat inactivation.

The protein content after ultrafiltration in the permeate increased with increasing hydrolysis time in all hydrolysates tested. This is due to the fact that the longer the hydrolysis time, the more the proteins were degraded as illustrated by the degree of hydrolysis. Hence the more peptides were produced and were susceptible to pass through the 10 kDa filter. Consequently, the protein content (actually peptide content) in the permeate increased due to higher degree of hydrolysis.

Table 2: Protein content and hydrolysis degree of Na-CN and WPC hydrolysed by different enzymes with increasing hydrolysis times, and separated by ultrafiltration (cut off value 10 kDa).

Enzymes	Blank enzyme		Before ultrafiltration				After ultrafiltration (permeate)				Hydrolysis degree
	Protein content				Protein content				Protein content		
	N	C (mg/ml)	Label	t (min)	N	C (mg/ml)	Label	t (min)	N	C (mg/ml)	
Trypsin	0,014	**0,89**	**CT5**	5	0,664	**42,36**	**CT5 UF**	5	0,387	**24,69**	**21,87**
	0,006	**0,38**	**CT30**	30	0,652	**41,60**	**CT30 UF**	30	0,51	**32,54**	**22,61**
	0,009	**0,57**	**CT60**	60	0,678	**43,26**	**CT60 UF**	60	0,518	**33,05**	**21,36**
	0,006	**0,38**	**CT180**	180	0,648	**41,34**	**CT180 UF**	180	0,551	**35,15**	**23,98**
	0,008	**0,51**	**CT360**	360	0,664	**42,36**	**CT360 UF**	360	0,565	**36,05**	**23,25**
	0,006	**0,38**	**WT5**	5	0,625	**39,88**	**WT5 UF**	5	0,227	**14,48**	**11,91**
	0,006	**0,38**	**WT30**	30	0,637	**40,64**	**WT30 UF**	30	0,328	**20,93**	**13,83**
	0,006	**0,38**	**WT60**	60	0,633	**40,39**	**WT60 UF**	60	0,37	**23,61**	**14,45**
	0,008	**0,51**	**WT180**	180	0,65	**41,47**	**WT180 UF**	180	0,439	**28,01**	**15,05**
	0,005	**0,32**	**WT360**	360	0,67	**42,75**	**WT360 UF**	360	0,466	**29,73**	**14,64**
Pancreatin	0,020	**1,28**	**CP5**	5	0,691	**44,09**	**CP5 UF**	5	0,658	**41,98**	**27,23**
	0,015	**0,96**	**CP30**	30	0,685	**43,70**	**CP30 UF**	30	0,648	**41,34**	**31,16**
	0,011	**0,70**	**CP60**	60	0,694	**44,28**	**CP60 UF**	60	0,653	**41,66**	**33,59**
	0,016	**1,02**	**CP180**	180	0,706	**45,04**	**CP180 UF**	180	0,68	**43,38**	**36,23**
	0,018	**1,15**	**CP360**	360	0,692	**44,15**	**CP360 UF**	360	0,676	**43,13**	**39,18**
	0,022	**1,40**	**WP5**	5	0,612	**39,05**	**WP5 UF**	5	0,266	**16,97**	**18,24**
	0,015	**0,96**	**WP30**	30	0,602	**38,41**	**WP30 UF**	30	0,587	**37,45**	**27,91**
	0,011	**0,70**	**WP60**	60	0,749	**47,79**	**WP60 UF**	60	0,439	**28,01**	**20,94**
	0,013	**0,83**	**WP180**	180	0,603	**38,47**	**WP180 UF**	180	0,563	**35,92**	**40,65**
	0,010	**0,64**	**WP360**	360	0,634	**40,45**	**WP360 UF**	360	0,6	**38,28**	**42,26**
A/O	0,032	**2,04**	**CAO5**	5	0,535	**34,13**	**CAO5 UF**	5	0,45	**28,71**	**33,87**
	0,025	**1,60**	**CAO30**	30	0,629	**40,13**	**CAO30 UF**	30	0,445	**28,39**	**29,98**
	0,027	**1,72**	**CAO60**	60	0,664	**42,36**	**CAO60 UF**	60	0,503	**32,09**	**29,67**
	0,025	**1,60**	**CAO180**	180	0,722	**46,06**	**CAO180 UF**	180	0,625	**39,88**	**31,71**
	0,030	**1,91**	**CAO360**	360	0,765	**48,81**	**CAO360 UF**	360	0,725	**46,26**	**35,76**
	0,011	**0,70**	**WAF5**	5	0,552	**35,22**	**WAF5 UF**	5	0,218	**13,91**	**26,47**
	0,007	**0,45**	**WAO30**	30	0,587	**37,45**	**WAO30 UF**	30	0,279	**17,80**	**28,14**
	0,012	**0,77**	**WAO60**	60	0,599	**38,22**	**WAO60 UF**	60	0,338	**21,56**	**29,90**
	0,008	**0,51**	**WAO180**	180	0,617	**39,36**	**WAO180 UF**	180	0,437	**27,88**	**34,04**
	0,008	**0,51**	**WAO360**	360	0,624	**39,81**	**WAO360 UF**	360	0,5	**31,90**	**41,08**

2.2. Sample analysis by CE-MS

2.2.1. Effect of ultrafiltration on peptide detection with CE-MS

Before testing the possibility to detect peptides in the real food matrix, hydrolysates were ultrafiltered. This process is a critical step to assure that the remaining intact proteins that escape hydrolysis are removed from the samples to analyse. Thanks to ultrafiltration, the method set up should efficiently detect peptides and not traces of residual intact proteins.

The effect of the ultrafiltration process on the detection of milk-derived peptides was assessed by analysing hydrolysate samples before and after ultrafiltration with CE-MS to determine if the peptide profile was affected by ultrafiltration. Peptides in ultrafiltrated hydrolysates might be too small to be properly identified by MS or might be retained in the filter preventing their collection in the filtrate. The spectrum acquired for whey proteins that have been hydrolysed with proteases from *Bacillus amyloliquefaciens* and *A. oryzae* is presented in the left panel in **Figure 5**, while the spectrum obtained for such a sample that underwent ultrafiltration prior to analysis is shown on the right. Milk-derived peptide sequences namely β-CN and αs1-CN indicated in **Figure 5** on the spectrum of CAO ultrafiltrated were also identified in the spectrum of CAO before ultrafiltration.

Globally, most peptides present in the unfiltrated hydrolysates were also detected in the permeate samples. The detectability of peptides in ultrafiltrated samples was even greater than in non-ultrafiltered samples. Many peptides originating from CNs could be identified in the hydrolysates after ultrafiltration suggesting that CE-MS was not affected by the ultrafiltration process.

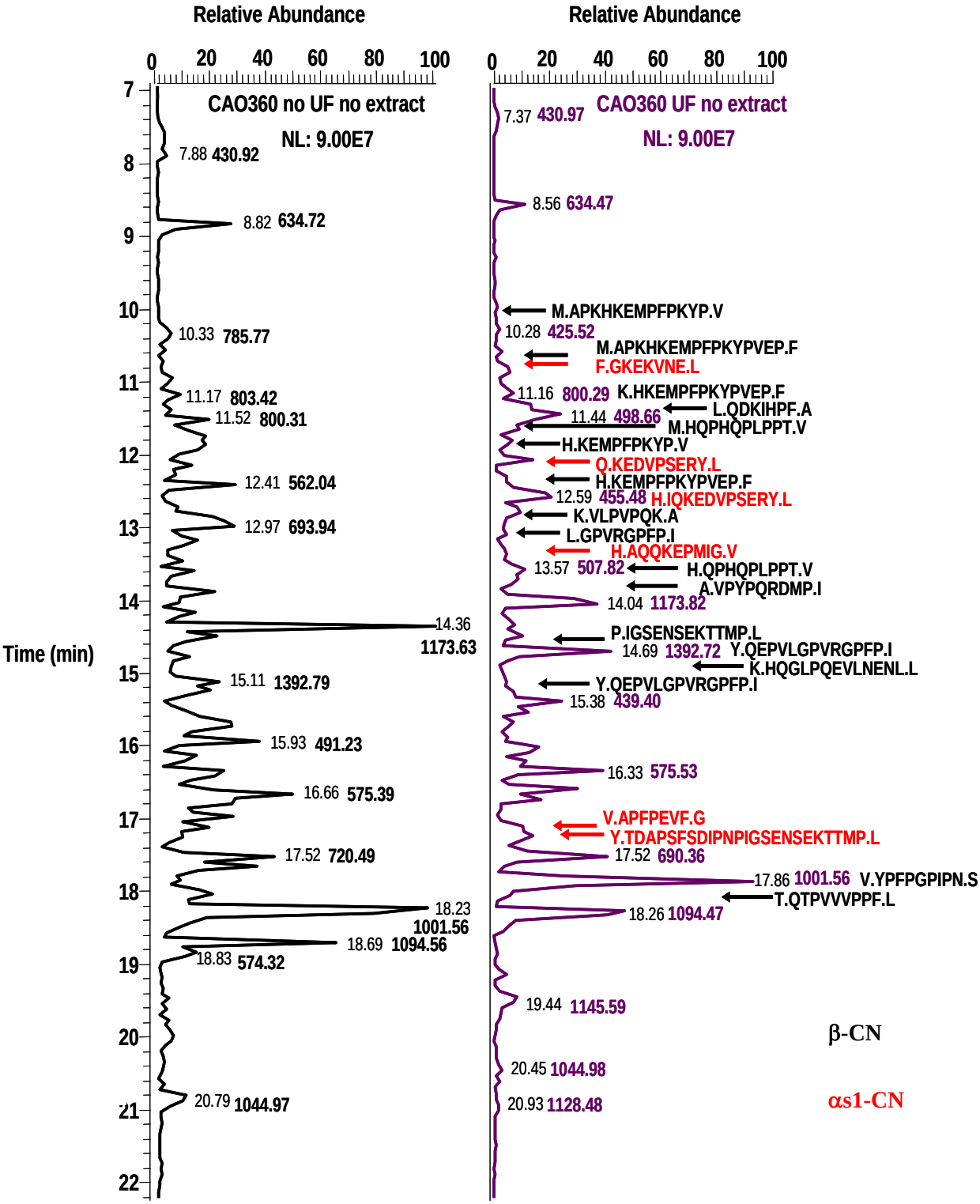


Figure 5: Effect of ultrafiltration process on CE-MS detection.

2.2.2. Feasibility of the CE-MS method to detect hydrolysates

To assess the feasibility of analysing hydrolysates spiked in a food matrix by CE-MS, it was necessary to test the efficiency of the extraction. Twelve of the 18 hydrolysates produced in 2007 were therefore spiked at 0.5% in acidified pure water before being extracted with cationic exchange MCX cartridge and analysed by CE-MS.

Table 3 summarises peptide detectability of each hydrolysate permeate tested by CE-MS.

Except for the hydrolysates produced after the proteolytic action of bromelain, milk-derived peptide sequences could be identified when analysed with Mascot Distiller and the Swissprot database (**Table 3**).

Table 3: Detection of milk-derived peptides in hydrolysates extracted from acidified water.

Proteins	Enzyme	Detection	Milk peptides detection
WPC	Trypsin (T)	yes	7 peptides β-LG
	Pancreatin (P)	yes	2 peptides β-LG
	Bromelain (B)	no	0 peptides
	B. Amyloliquefaciens (A)	yes	6 peptidesβ-LG
	A. Oryzae (O)	yes	3 peptidesβLG
	A+O	yes	8 peptidesβ-LG
CN	Trypsin	yes	6 peptidesαs-1 CN
	Pancreatin	yes	5 peptidesβ-CN, 5 peptidesαs-1 CN
	Bromelain	no	0
	B. Amyloliquefaciens	yes	3 peptidesαs-1 CN; 2 peptidesβ-CN
	A. Oryzae	yes	2 peptides β-CN
	A+O	yes	8 peptidesβ-CN; 3 peptidesαs1-CN

These results demonstrate that both the extraction by cationic exchange and the identification of milk-derived peptides by CE-MS were feasable. Consequently, the analysis of food matrices (orange juice and multi-vitamin juice) spiked with hydrolysates could be investigated.

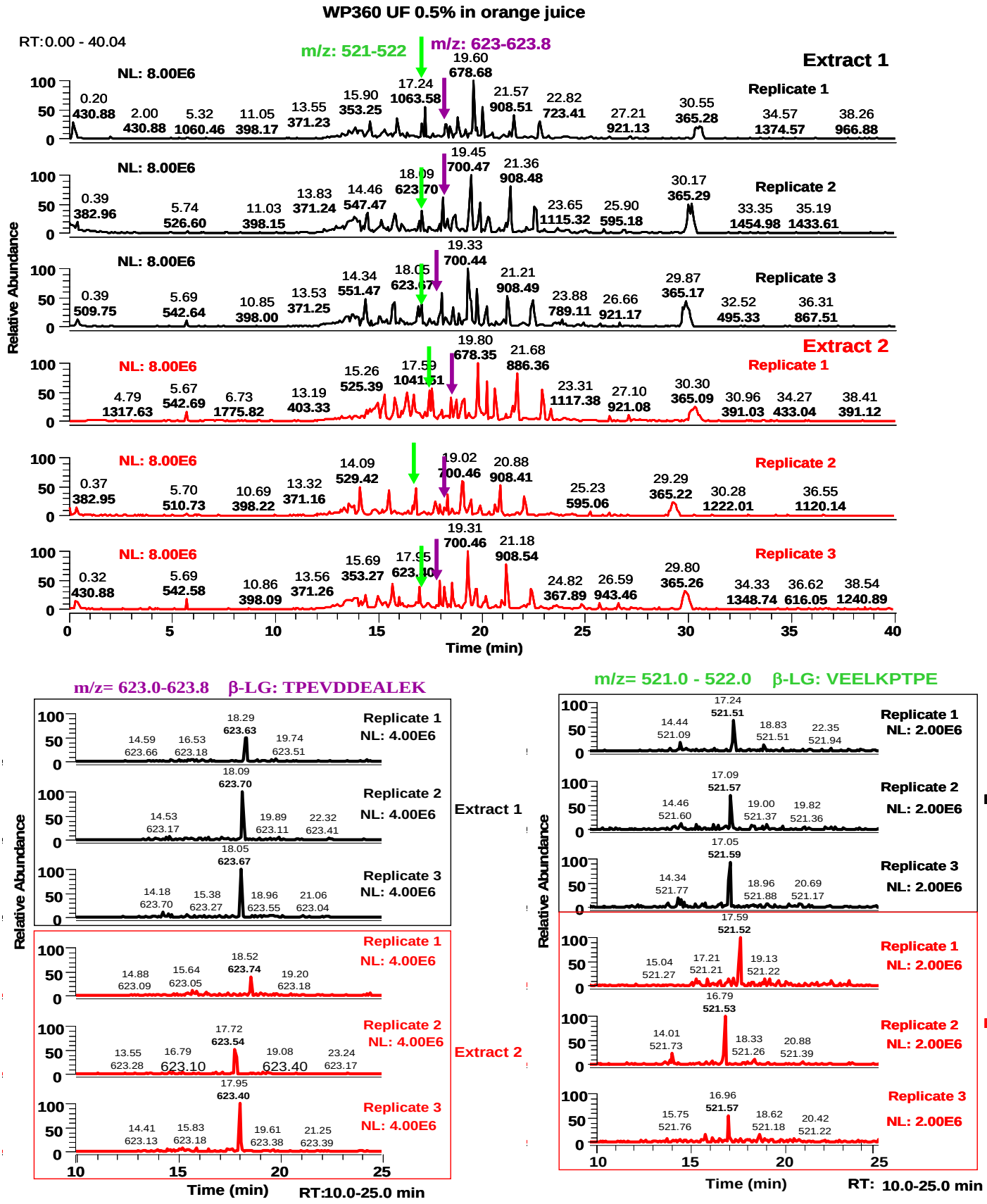
The following spiking experiments were performed on new batches of hydrolysates produced in bigger volumes after demonstrating the capacity to detect peptides after extraction with the SPE cartridges.

2.2.3. Repeatability

The repeatability of the CE-MS method was assessed by replicating the injections of two different extracts. An example of the repeatability of the CE-MS method is illustrated by following the detection of whey protein hydrolysed with pancreatin spiked at 0.5% into orange juice (**Figure 6**).

Mass spectra obtained from extract 1 (in black) run in triplicate show a real similarity despite small shifts in retention time. The same observation can be made for the replicates of extract 2 (in red), that show spectral homology. Furthermore, comparison between spectra acquired with extract 1 at 0.5% with extract 2 does not reveal significant differences: the detected peptides were identical for all samples. The repeatability of the CE-MS method was confirmed by looking more specifically to those two ions m/z 623 and m/z 521 respectively highlighted by colored arrows on the upper spectra. These ions corresponding to 2 peptides from β-LG (lower part of **Figure 6**) were present in each spectra of the extracted orange juice with comparable intensities and retention times.

Figure 6: Repeatability of CE-MS method tested in two extracts of orange juice spiked with 0.5% WP360 UF.



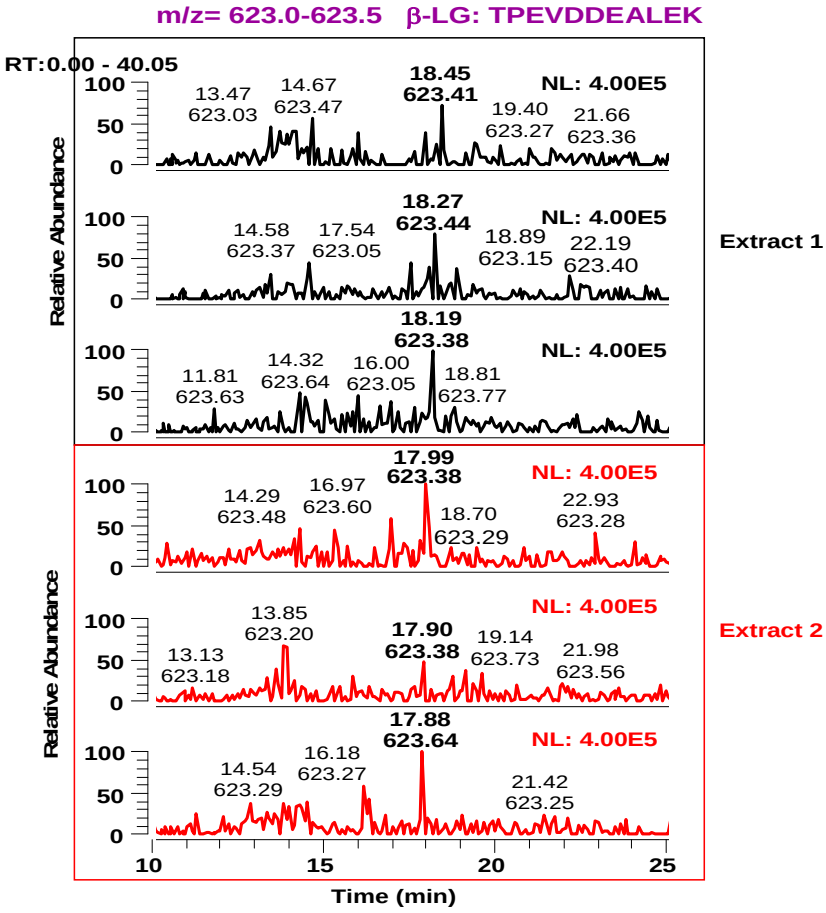
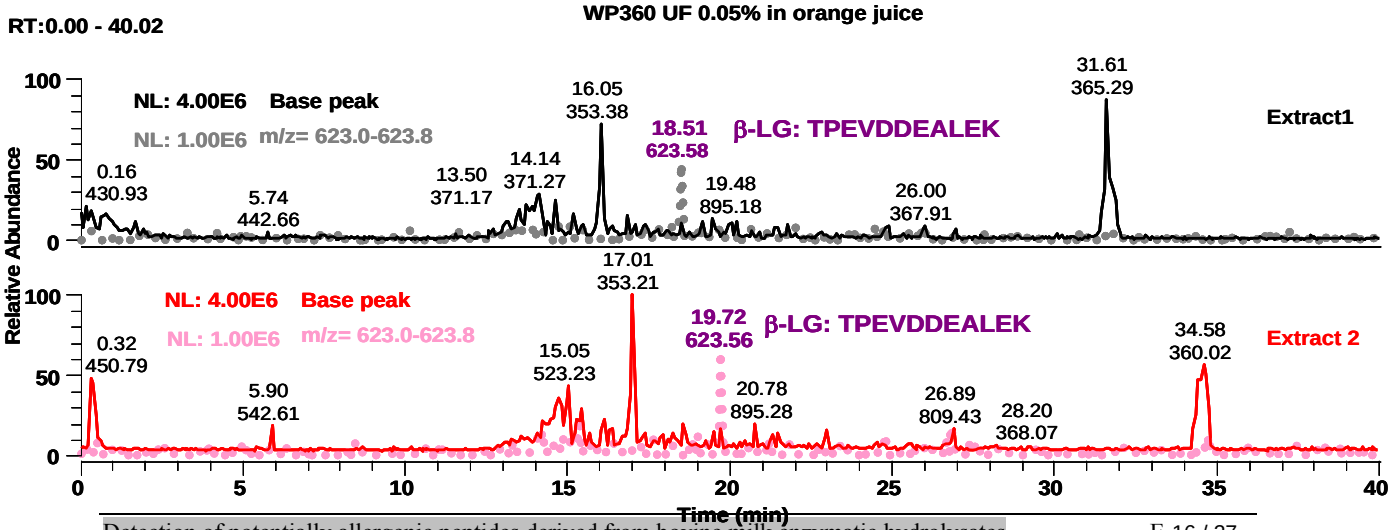


Figure 7: Repeatability of the detection of WP peptide marker (m/z 623) in 0.1% spiked orange juice.

Detectability declined more and more at 0.05% spiking level in which the sequences of the peptide "markers" shown in **Figure 6** were not retrieved from the Swissprot database. However, even at 0.05% spiking level, the mass spectra were comparable between the spectra. Furthermore, the presence of the peptide marker m/z 623, even if not found after the sequence search, can be visualised in **Figure 8** for both extracts. This is highlighted by overlapping the amplified spectra of this m/z 623 ion (in dotted lines) to the total mass spectra (in continuous lines). The position of this peptide is comparable in both extracts attesting of the repeatability of the CE-MS at this spiking level.

Figure 8: Detection of WP peptide markers in 0.05% spiked orange juice.



2.2.4. Sensitivity

To estimate the sensitivity of the CE-MS method, different spiking levels were realised in orange juice respectively 0.01%, 0.05%, 0.1%, 0.5% (v/v). The different spiking levels were obtained by preparing a starting solution of juice spiked with 1% hydrolysate (v/v), which was serially diluted with blank matrix. Hydrolysates obtained with trypsin were used for this purpose. The tryptic digests served, as mentioned previously, as the reference to determine the sensitivity of the method since the peptides emerging from this proteolysis were well characterised and identified in contrast to the peptides obtained with the other enzymes used, which possess a broader specificity of action.

As expected, when decreasing the amount of WT added to the orange juice, the intensities of milk-derived peptide peaks decreased resulting in mass spectra patterns coming closer and closer to the mass spectrum of the blank (**Figure 9**).

The detection of milk-derived peptide peaks decreased drastically at spiking levels of 0.05 and 0.01% and at the level of 0.01%, no relevant milk-derived peptide sequences could be extracted from the database search (**Table 4**).

Figure 9: Sensitivity of the CE-MS method for the detection of WT spiked at different levels in orange juice and extracted by cation exchange cartridge.

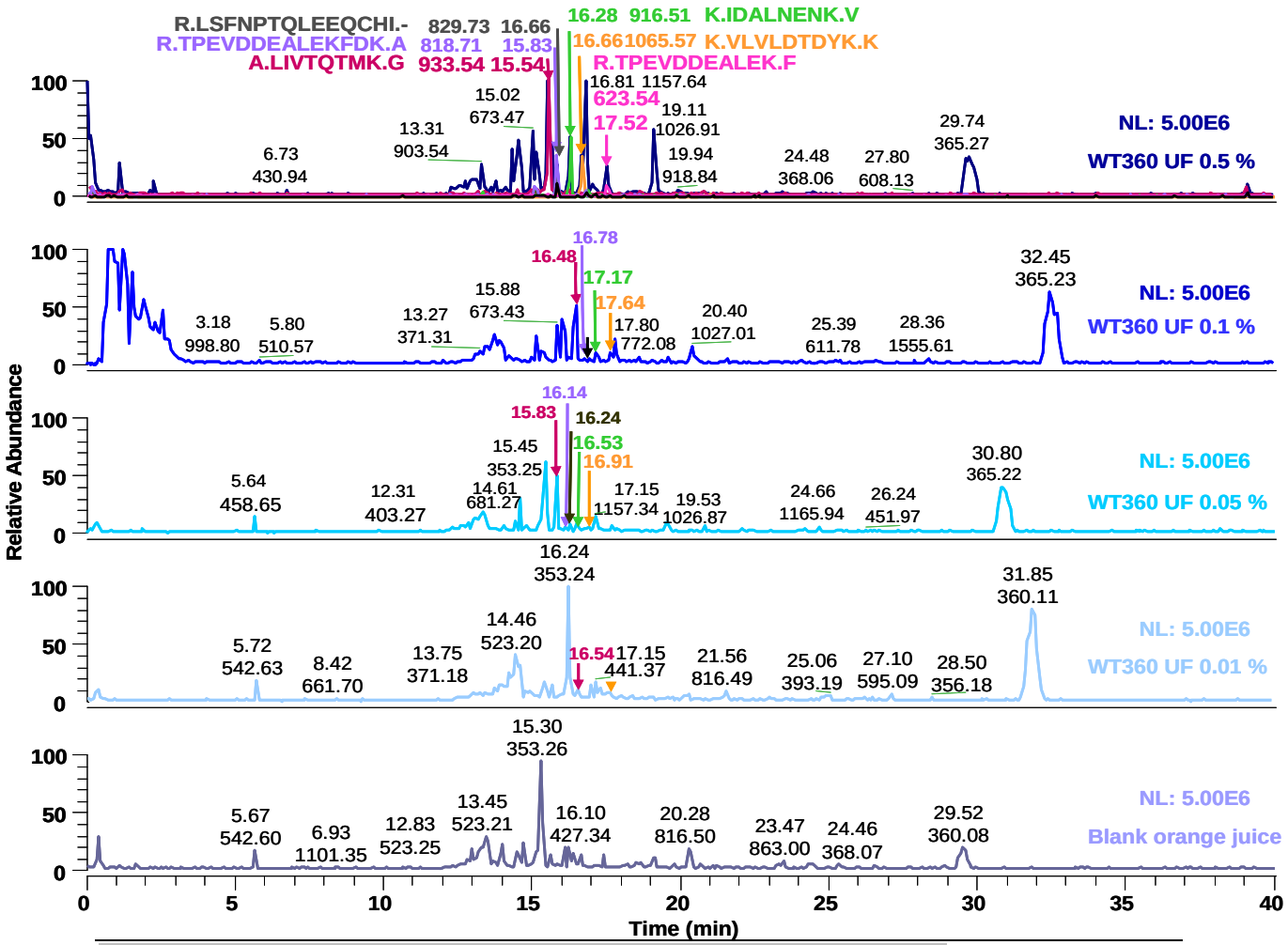


Table 4: Peptide sequences identified in orange juice spiked with WT360 UF.

Spiking levels	m/z Observed	Mass (expected)	Mass (calculated)	Delta (mass difference)	Score	Peptide sequences
0,50%	916.3691	915.3618	915.4661	-0.1043	26	K.IDALNENK.V
	933.4303	932.4230	932.5365	-0.1135	13	A.LIVTQTMK.G
	1065.4702	1064.4629	1064.5754	-0.1125	32	K.VLVLDTDYK.K
	623.2142	1244.4139	1244.5772	-0.1633	63	R.TPEVDDEALEK.F
	814.2261	1626.4376	1626.8577	-0.4200	48	M.AASDISLLDAQSAPLR.V
	818.2992	1634.5838	1634.7675	-0.1837	68	R.TPEVDDEALEKFDK.A
	829.7335	1657.4524	1657.7770	-0.3246	45	R.LSFNPTQLEEQCHI.-
0,10%	419.0940	836.1735	836.4691	-0.2955	29	K.ALPMHIR.L
	933.4370	932.4297	932.5365	-0.1068	23	A.LIVTQTMK.G
	818.2409	1634.4672	1634.7675	-0.3003	35	R.TPEVDDEALEKFDK.A
	829.7111	1657.4077	1657.7770	-0.3692	17	R.LSFNPTQLEEQCHI.-
	771.6287	2311.8642	2312.2515	-0.3873	50	R.VYVEELKPTPEGDLEILLQK.W
0,05%	818.2157	1634.4168	1634.7675	-0.3507	35	R.TPEVDDEALEKFDK.A
	829.6948	1657.3751	1657.7770	-0.4019	25	R.LSFNPTQLEEQCHI.-
0,01%	-	-	-	-	-	-

However, when following specific ions (as presented in **Figure 10**), the presence of those peptides can be highlighted even if their intensities (reduction of the NL value indicated on the right hand of the graph under the sample description) decreased as seen in **Figure 10**.

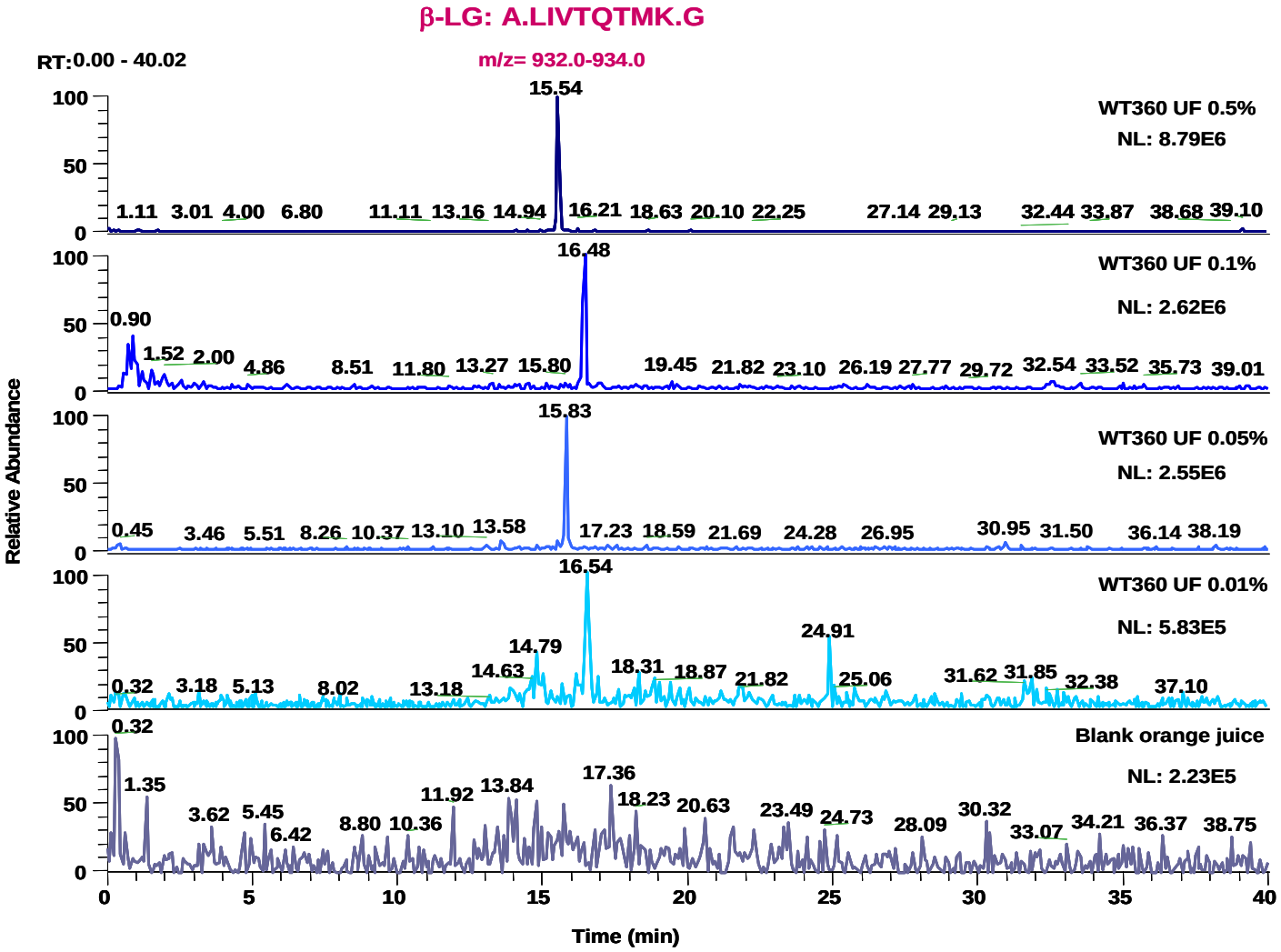


Figure 10: "Tryptic peptide" marker for WPC followed in MS spectra for orange juice spiked with different amounts of hydrolysed WPC.

2.2.5. Matrix effects

2.2.5.1. Orange juice

The identification of milk-derived peptides in orange juice was successful for both hydrolysed-whey proteins and caseins-derived peptides in an orange juice matrix. The sequences of peptides identified in orange juice extracts spiked with CP360 UF are presented in **Table 5**.

While peptide sequences emerging from the digestion with pancreatin could be detected to comparable detection limits as tryptic digests, the detection of A/O hydrolysate is less performant (detection up to 0.1% spiking level). Those differences are likely linked to the fact that A/O treatment results in the emergence of peptides of smaller size than tryptic or pancreatic digests that cannot be detected and identified in CE-MS. Parallely, the peptides formed during hydrolysis possessed different properties and consequently might have influenced their capacity to be efficiently extracted with the cationic

exchange SPE method. This suggests that the extraction method should be looked at carefully and improved to potentially improve the recovery of A/O peptides.

Table 5: Milk-derived peptide sequence identified in orange juice extracts spiked with CP360 UF.

Hydrolysate	spiking level	Protein	Charge	m/z Observed	Mass expected	Delta (mass difference)	Score	Peptide sequence
CP	0,50%	β-CN	1	748.2268	747.2195	-0.1431	15	K.EMPFPK.Y
			1	838.3017	837.2945	-0.1652	26	Y.QEPVLGPV.R
			2	641.6830	1281.3515	-0.2773	48	W.MHQPHQPLPPT.V
			2	650.5834	1299.1523	-0.5340	41	Q.SLVYPFPGPIPN.S
			2	969.3339	1936.6532	-0.3086	20	M.HQPHQPLPPTVMFPPQS.V
			2	1018.8733	2035.7320	-0.2982	6	M.HQPHQPLPPTVMFPPQSV.L
			3	723.2372	2166.6897	-0.3810	18	W.MHQPHQPLPPTVMFPPQSV.L
		αs1-CN	1	962.4150	961.4077	-0.0833	28	F.VAPFPEVFG.K
			2	687.0629	1372.1112	-0.5398	21	Y.TDAPSFSDIPNPI.G
	0,10%	β-CN	1	751.2488	750.2415	-0.1174	21	K.YPVEPF.T
			1	838.2797	837.2724	-0.1872	28	Y.QEPVLGPV.R
		αs1-CN	1	962.3110	961.3037	-0.1872	23	F.VAPFPEVFG.K
			1	687.0539	1372.0933	-0.5578	25	Y.TDAPSFSDIPNPI.G
			1	758.2091	757.2019	-0.1992	34	F.VAPFPEV.F
			1	650.1926	1298.3707	-0.3527	17	K.YKVPQLEIVPN.S
	0,05%	β-CN	1	751.2555	750.2483	-0.1106	23	K.YPVEPF.T
			1	838.2944	837.2872	-0.1725	19	Y.QEPVLGPV.R
			1	1100.4135	1099.4062	-0.1640	14	L.VYPFPGPIPN.S

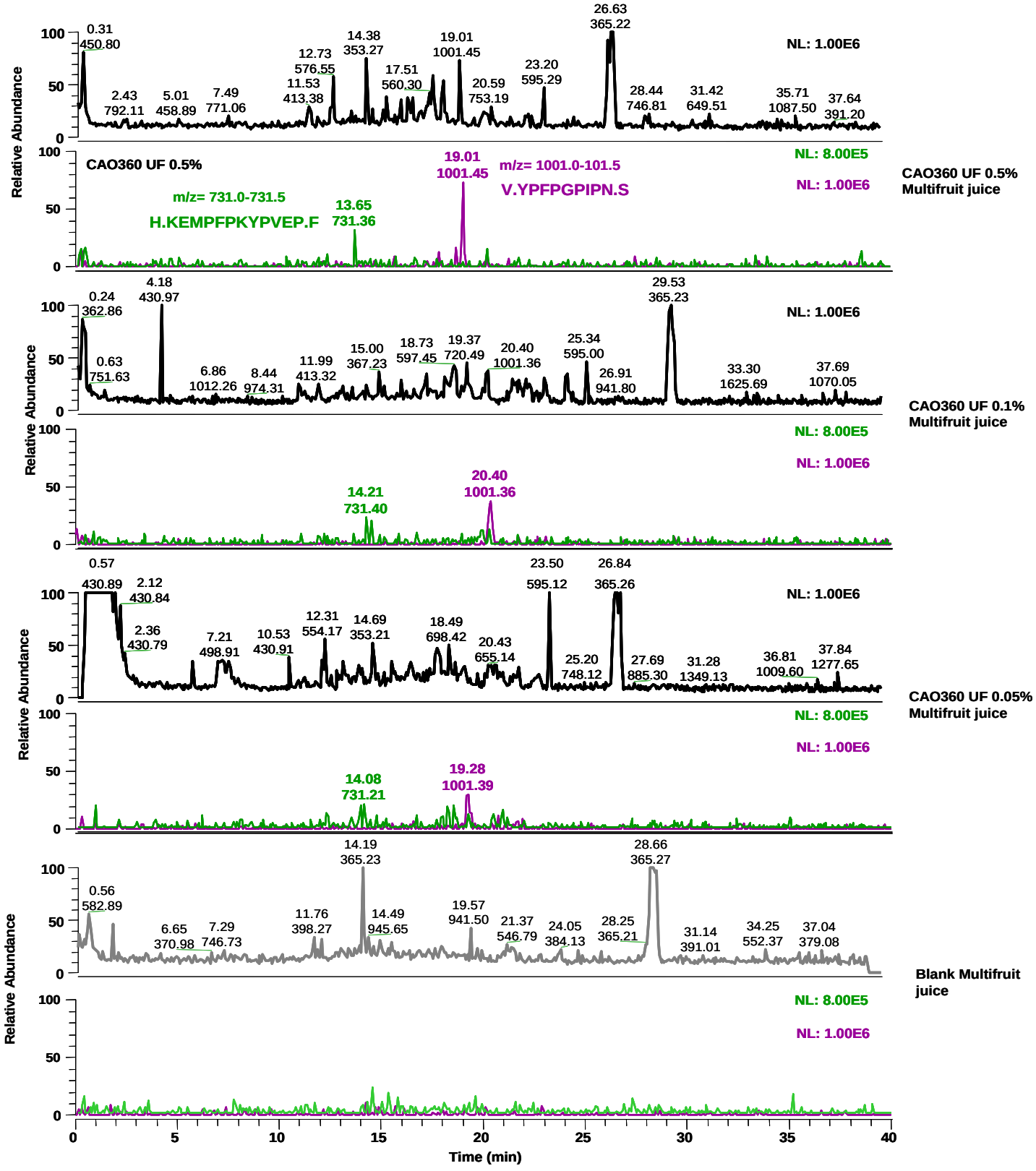
2.2.5.2. Multifruit juice

The detection of milk-derived peptides was also investigated in multifruit juice to determine the suitability of the CE-MS method for a slightly more complex food matrix. The determination of the presence of milk-derived peptides performed in the same manner as orange juice was also successful in the multifruit juice matrix. **Figure 11** shows an example of the identification in the mass spectra of CAO360 UF-derived peptides extracted from multifruit juice spiked at different levels. Several peptides derived from β-CN could be identified in the recorded mass spectra (in black). The profile of the full mass spectra (in black) evolved accordingly to the reduction of the CAO amount in multifruit juice i.e. the separation worsened and the intensities of the peaks decreased until the spectra become similar to the spectrum of the blank.

This is better visualised when filtering the mass spectra to follow 2 specific ions, respectively m/z 731 and 1001, seen in the colored intercalated mass spectra. Those two ions identified as peptides derived from β-CN are clearly detected at 0.5% spiking level. The intensity of the peaks corresponding to these ions, decreased while reducing the quantity of CAO added to the multifruit juice.

Milk-derived peptides could be identified in the multifruit juices spiked with all other types of hydrolysates i.e. pancreatic, tryptic digests from both WPC and CNs except WAO. However, the identification of milk-derived peptides for WAO in multifruit juice could be, solved when changing the sample preparation (anionic exchange extraction) as performed for WAO spiked into a protein enriched drink in which milk-derived peptides could not be detected with cationic exchange extraction but were identified when extracted with anionic exchange extraction.

Figure 11: Detection of CN peptides extracted from multifruit juice spiked with CAO 360 UF.



2.3. Detectability of milk-derived peptides in juices by CE-MS

When considering that the detection of peptides in extracted samples was mainly effective at 0.5 and 0.1%, it can be concluded that the method so far can detect peptides mainly in the range of 30-230 ppm for matrices like multivitamin juices (**Table 6**). The ppm values of hydrolysates added in the juice resumed in **Table 6** were estimated from the protein concentrations presented in **Table 2**.

Table 6: Concentration of milk-derived peptides in juices (in ppm).

Hydrolysates	C(mg/ml)	ppm	C (ppm) spiked in the juices			
			0,01%	0,05%	0,10%	0,50%
CT360 UF	36,05	36047	4	18	36	180
WT360 UF	29,73	29731	3	15	30	149
CP360 UF	43,13	43129	4	22	43	216
WP360 UF	38,28	38280	4	19	38	191
CAO360 UF	46,26	46255	5	23	46	231
WAO360 UF	31,90	31900	3	16	32	160

However, it has to be taken into account that the detection of peptides is more complex than detection of intact proteins. Indeed the concentration determined by nitrogen content gives an estimation of the peptide content but provides no information about the abundance of the individual peptides. Since peptides of 2-4 amino acids are difficult to identify (cannot be milk-specific), there is a bias towards those peptides that can indeed be detected and identified. The abundance of the different peptides is an important parameter in the potential detection in food matrices.

Furthermore, the extraction method is critical because the peptides may react differently towards the SPE phase as well as they may have less affinity for this phase than interfering proteins present in the matrix. Consequently, a number of peptides will not be retained and will be lost for further detection. The extraction method might have to be improved in order to enhance the potential recovery of peptides.

Additionally, an important aspect that has to be considered is the low quantities of sample injected in the CE-MS capillary i.e in the pg level (**Table 7**).

Table 7: Quantities of peptides injected in the separation systems to be analysed by MS

Hydrolysates	Amount of peptides injected in CE-MS system (pg)			
	0,50%	0,10%	0,05%	0,01%
CT360 UF	19	4	2	> 1
WT360 UF	15	3	2	> 1
CP360 UF	22	4	2	> 1
WP360 UF	20	4	2	> 1
CAO360 UF	24	5	2	> 1
WAO360 UF	16	3	2	> 1

To improve the detectability of milk-derived peptides in juices, the sample should be more concentrated before being analysed by CE-MS.

Another determinant parameter in the efficiency of the identification of peptides is inherent to the sequence search software. In the present study, the analysis was performed with the Swissprot database. Furthermore the fact that the enzymes used are not in the enzyme list of the software, leads to a considerable increase of the time of analysis, an increase of the threshold of strict identification of a specific sequence, and often to a reduction of the number of sequences identified. The implementation of a distinct database reduced to milk proteins should improve the detection of milk-derived peptides in the tested samples.

3. CONCLUSION

The CE-MS method is a promising technique for the detection of milk-derived peptides in food matrices. Indeed, such peptides obtained after hydrolysis of milk proteins and subsequent ultrafiltration could be detected and identified in fruit juices, when using CE-MS after solid phase extraction. Currently used methods based on ELISA or PCR fail to do so, as demonstrated in our exploratory project of 2007. The CE-MS method set up in the orange juice matrix showed a good repeatability and sensitivity down to concentrations of 15-23 ppm. It was demonstrated that milk-derived peptides could be efficiently identified in a more complex matrix than orange juice namely a multifruit juice. Furthermore, milk-derived peptides emerging from different enzymatic treatments than trypsin could also be extracted and identified from both juices (orange and multifruit). So far the best results for the CE-MS method in terms of sensitivity are reached using tryptic digests, probably due to the fact that mass spectra and fragmentation patterns recognised in the Swissprot database are well defined.

Several routes of optimisations might be attempted in future to improve the detection in different matrices and types of hydrolysates. The sensitivity is always one of the key factors and in the current CE-MS method the enhancement of the sensitivity could be obtain by avoiding dilution of the samples during the analysis due to the sheath liquid. The implementation of a new interface “sheath liquid free” could improve the signal.

Further attention might be given to the sample preparation with the optimisation of the extraction and clean up method. The sample treatment of the spiked sample is a limiting factor for the further analysis in CE-MS and should be improved to maximise the recovery of the peptides and elimination of the matrix compounds.

After perfecting the sample preparation method, the CE-MS method should be tested for its ability to detect and identify milk-derived peptides from more complex beverages such as protein enriched juices. Further on the CE-MS method might be applied for the identification of peptides from commercially available milk protein hypoallergenic formulae.

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ANNEXES

Annex 1: Commercial juices composition.

Product	**Minute Maid Orange juice**	**MultiVitamin Light**
	Orange juice made from concentrated orange juice 100% orange juice	Nectar of 12 fruits with sweeteners.
Packaging	1L	1L
Composition	100% pur juice naturally enriched in Vitamin C. Contains only natural sugars present in the fruits.	Minimal fruit content: 50% Made from concentrate of 12 fruit juices: (orange, pineapple, apple, grape, passion fruit, pear, lemon juices) and concentrate of fruit puree (banana, nectarine, mango, guava, papaya), water, sweeteners (Na cyclamate; Na saccharine) and vitamins (C, E, B6, B12, Niacin, Pantothenic acid, b-caroten (proVitamin A), riboflavin, biotin, thiamine)
Energetic Value	195 kJ/ 46 Kcal	95 KJ/ 22Kcal
Proteins (g/100 ml)	0.7	0.2
Glucides (g/100 ml)	11	5
sugars (g/100 ml)	11	4.99
Lipids (g/100ml)	0	<1
Saturated Fatty acids (g/100 ml)	0	<0.02
Sodium (g)	0	<0.02
Alimentary fibers (g)	0	0.1
Vitamine C (mg/100 ml)	30 mg	9
Vitamine E		1.5
Vitamin B6		0.2
Niacin		2.7
Panthothenic acid		0.9
Riboflavin		0.24
Thiamin		0.21
Biotin		0.02
Vitamine A (µg)		120
vitamin B12 (µg)		0.15

EUROPEAN COMMISSION
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Institute for Reference Materials and Measurements
Food safety and quality

Exploratory study on the application of metabolomic and metabonomic approaches for the discrimination of organically and conventionally grown carrots

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1. SUMMARY

A growing market share and premium price of organically grown products, as well as the priorities stipulated in the European Action Plan for Organic Food and Farming, are the background of the need for developing analytical methods which aim to discriminate conventionally and organically grown agricultural products. In our exploratory research study we have focused on the plant defence system of carrots as the potential source of discrimination. Metabolome fingerprint comparison was used to reveal potential differences between organically and conventionally grown carrots, which were selected as a model biological system for this study. Liquid chromatography was used for determining the metabolomic changes caused by chemical and biological perturbations in both plant materials. The plant's priming procedure coupled with microbiological testing was also evaluated for its discrimination power.

Based on the presented results we conclude that the following approaches had an increasing power to discriminate organically from conventionally grown carrots: i) priming plants with BABA and microbiological testing, ii) finger printing of the metabolome based on ultra performance liquid chromatography coupled to mass spectroscopy, iii) priming with chitosan and measuring the amount of an unknown compound eluting from an HPLC system at a retention time of 8.6 min, and finally iv) the best results showing no overlap of groups was observed via the presence of an unknown compound with retention time 8.6 min, which could be observed after microbiological inoculations.
However, an important future task would be to identify the unknown component.

2. INTRODUCTION

2.1. Organic and conventional agriculture – discrimination required

European Council Regulation (EC) No 834/2007 defines requirements for organic production in general. In case of organic farming the fertility of the soil should be maintained by multiannual crop rotation including legumes and other green manure crops. Application of livestock manure or organic material should be preferably composed from organic production. The prevention of damage caused by pests, diseases and weeds shall rely primarily on the protection by natural enemies, the choice of species and varieties, crop rotation, cultivation techniques and thermal processes.

Due to the fact that quantifiable parameters are missing the control of organic agricultural production is based on inspection systems.

Organically grown agricultural products generally experience a premium price over conventional products explaining why fraud cases can appear. To prevent fraud and to support the already existing inspection systems, analytical methodology offering quantifiable data for the discrimination of organically and conventionally grown agricultural products procedures is needed. A 5-year project ADAB – *Authentification de Denrées Alimentaires issues de l'Agriculture Biologique* at JRC, IRMM, Geel screened 16 various analytical methods for their potential in such discrimination.

The present study is an extension of this project focusing on plant defence systems and potential stress factors, as reflected in selected phenolic acids determination and adoption of metabolomic and metabonomic procedures.

2.2. Phenolic acids in the plant defence system

Plant defence systems are complicated mechanisms (Estabrook and Yoder 1998; Bruce and Pickett 2007) in which, amongst others, flavonoids and phenolic acids play an important role (Bennett and Wallsgrove 1994; Estabrook and Yoder 1998; Kawano, Furuichi et al. 2004). Phenolic acids are intermediates in biological pathways (beginning with phenylalanine and tyrosine) in lignin formation (Moore and JUNG 2001) to reinforce cell walls on the place of microbiological invasion (Bennett and Wallsgrove 1994; Estabrook and Yoder 1998; De Ascensao and Dubery 2000; Moore and JUNG 2001; Kawano, Furuichi et al. 2004). Phenolic acids are also bound to cellulose and afterwards the modified cellulose is less digestible for microorganisms (Bennett and Wallsgrove 1994). Salicylic acid is involved in Systemic Acquired Resistance (Kawano, Furuichi et al. 2004), where this compound has a role of "bad news messenger". Infected plants release salicylic methyl ester from the leaves, which triggers the immune system in plants in close neighbourhood after its hydrolysis to the free acid. Phenolic acids (mainly chlorogenic acid) play an important role in allelopathy, i.e the plant to plant competition for space for life (Mulabagal and Hsin-Sheng 2004).
Phenolic acids are present in plants in free form or bound in various forms mostly as esters (Fallico, Lanza et al. 1996; Mulinacci, Innocenti et al. 2005). Six different binary bindings of ferulic acid (Waldron, Parr et al. 1996), ferulic acid glycosides as well as

diferulic acid glycosides have been reported up to the tetramer. Binary binding also is known to occur for sinapic acid (Bunzel, Ralph et al. 2004; Allerdings, Ralph et al. 2005), and caffeic acid has been detected as a trimer, a tetramer and in other forms (Jiang, Lau et al. 2005).

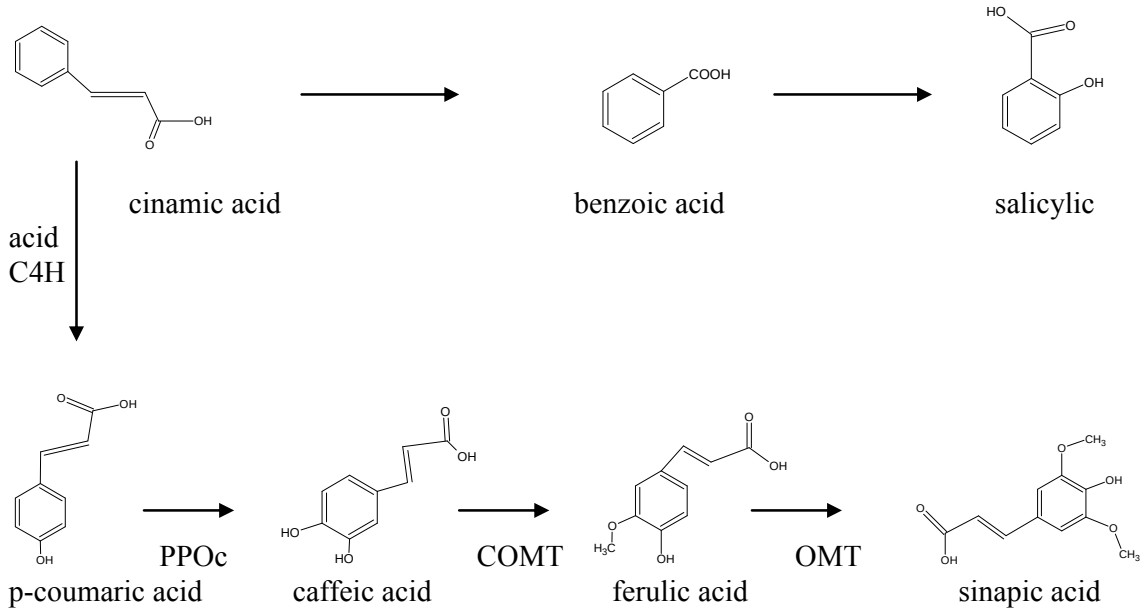


Figure 1. Biological transformation pathway of some phenolic acids.

Enzymes:
C4H … cinamate 4-hydroxylase
PPOc ….. cresolase activity polyphenol oxydase
COMT ….cafeat-o-methyl transferase
OMT ….. o-methyl transferase

It is well known that the stability of phenolic acids is rather limited. In the presence of certain enzymes they are transformed (Sarni-Machado and Cheynier 2006) (Figure 1).

Within the framework of the basic project (the 5 year long ADAB study) we have already determined phenolic acids in four crops – carrot, potato, winter wheat and horse bean. In all crops, plant to plant variability was higher than the uncertainty of measurement, especially in carrot. A very high microenvironment impact was observed (Figure 2). Altogether, this means the discrimination between organically and conventionally grown carrots is difficult, even when strong trends are observed.

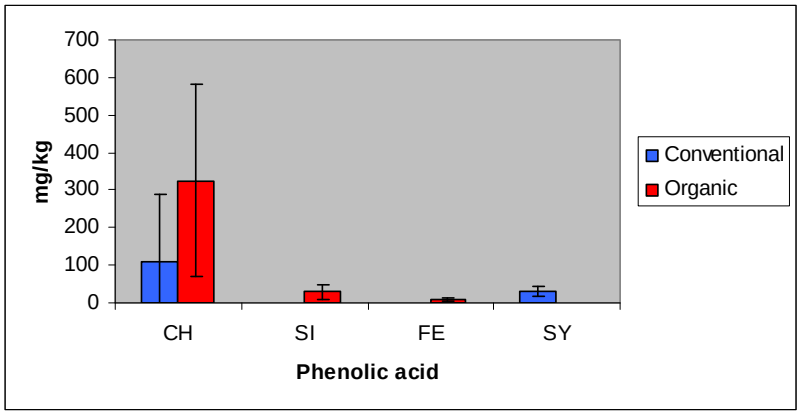


Figure 2. Determination of free phenolic acids in carrots from 2005 harvest season.
CH – chlorogenic acid, SI – sinapic acid, FE – ferulic acid, SY – syringic acid.

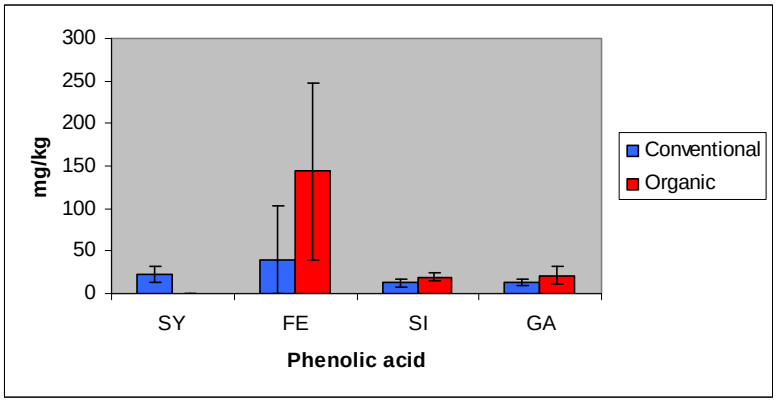


Figure 3.Determination of phenolic acids after acidic extraction in carrots from 2005 harvest season.
SY – syringic acid, FE – ferulic acid, SI – sinapic acid, GA – gallic acid.

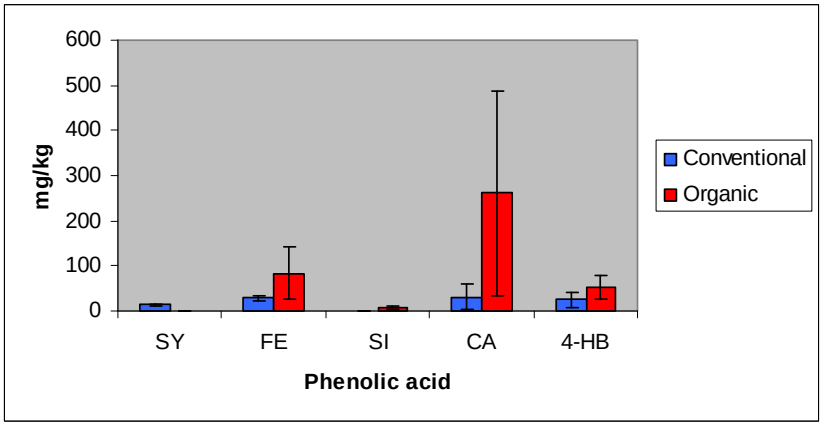


Figure 4. Determination of phenolic acids after alkaline extraction in carrots from 2005 harvest season.
SY – syringic acid, FE – ferulic acid, SI – sinapic acid, CA – caffeic acid, 4-HB – 4-hydroxy benzoic acid.

Contrary to conventional agriculture organic agriculture should not use off farm materials. Synthetic chemical pesticides are avoided in organic agriculture. This could be reflected in the variability of the metabolome composition between these two agricultural production systems. We assume that the plant defence system in conventionally grown plants will show different responses after applying suitable perturbation (stressing) than the organically grown one. The plant immunity system is assumed to be affected by plant protection products in conventional production systems.

2.3. Priming of plants

The infection of plants by necrosis-inducing pathogens or the colonization of plant roots with certain beneficial microbes induces a unique physiological state called priming (Conrath, Beckers et al. 2006). The primed state can also be induced by treatment of plants with natural and synthetic compounds. Priming by chemicals for potentiated responses to biotic stress can be achieved with the non-proteinogenic amino acid beta-amino butyric acid (BABA). This compound is known as a potent agent for induction of resistance in plants against microbial pathogens (Conrath, Beckers et al. 2006; Beckers and Conrath 2007).
In case of carrots chitosan was shown to have a priming ability as well (Cheah, Page et al. 1997; Molloy, Cheah et al. 2003).
Primed plants have a higher resistance against microbial attack (Conrath, Beckers et al. 2006). In some cases the effect of priming is comparable to pesticides application. This is valid for the case of moderate invasion of pathogenic microorganisms.
In our project we have applied priming with BABA, chitosan and in addition a reduced invasion of pathogenic *Sclerotinia sclerotiorum* was tested.

2.4. Metabolomic study

Recent advances in technology have brought about a revolution to the manner in which biological systems are visualized and queried. Systems biology encompasses a holistic approach to the study of biology and the objective is to simultaneously monitor all biological processes operating as an integrated system (Sumner, Mendes et al. 2003). Through the study of systems, one can begin to visualize how individual pathways or metabolic networks are interconnected. Functional elucidation of genes (functional genomics) can be pursued through the systematic perturbation of gene expression followed by quantitative and qualitative analyses of gene expression products including mRNA, proteins, and metabolites (Oliver, Nikolau et al. 2002). At the analytical level, both functional genomics and systems biology rely on the comprehensive profiling of large numbers of gene expression products. These approaches are commonly referred to as transcriptomics (Holtorf, Guitton et al. 2002), proteomics (Jorrin-Novo 2009), and metabolomics (Fiehn and al. 2000; Allwood, Ellis et al. 2008). The concept of profiling the metabolome has not been as frequently attempted as its parallel ‘‘-omics’’ counterparts even though the necessary technologies have a much longer history. This is primarily due to the technical complexity of metabolomics itself. Nevertheless, exponential growth is being observed in the use of metabolomics in recent years, and the impact of this approach is now being felt in many areas of biology. The components of the metabolome can be considered the end products of gene expression and thus define

the biochemical phenotype of a cell or tissue. The comprehensive quantitative and qualitative analysis of all metabolites within a cell, tissue or organism is a very ambitious goal and is still far from reality for any system. Many responses involving altered gene expression, particularly those of plants to environmental stimuli, result in qualitative changes in metabolite pools, and therefore qualitative identification of the metabolites will be critical. At the same time, some genetic modifications or environmental responses may result only in temporal or spatial changes in metabolite concentrations. Thus, accurate and reproducible quantitative methods are also necessary. The aim of our investigation was to apply the metabolomic approach in order to highlight possible metabolic differences between carrots (*Daucus carota* L.) grown in different agricultural systems, namely organic versus conventional. Two different environments were selected in order to monitor also this possible source of variability.

Metabolomics originates from metabolite profiling. During the early 1980s, results from the application of HPLC (High Performance Liquid Chromatography) and NMR (Nuclear Magnetic Resonance) for metabolite profiling (Nicholson et al. 1984) began appearing in the literature. Central to the metabolomic process are a variety of chemical profiling technologies. It is generally accepted that a single analytical technique will not provide sufficient visualization of the metabolome and, therefore, multiple technologies are needed for a comprehensive view (Hall et al. 2002; Sumner, Mendes et al. 2003). Moreover, metabolomics measurement will rely heavily upon bioinformatics for the storage, retrieval, and analysis of large datasets. Changes in metabolite levels may be dramatic or subtle. The dramatic changes will be easily recognized; however, subtle changes will require statistical processing to determine whether or not the observed changes are significant (Miller and Miller 2000). Statistical approaches require careful experimental design including replicate sampling, replicate analyses and application of statistical tests. In our study, two of the most valuable spectroscopic tools, NMR spectroscopy and Liquid Chromatography-Mass Spectrometry (LC-MS), were applied for the acquisition of metabolic profiles of in house prepared carrot extracts. The acquired data were then processed using specific software for data handling as well as for the successive chemometric analysis as presented in the next section.

2.5. Metabonomics

Metabonomics is defined as "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification". Metabonomic applies the same principles as are used in metabolomic studies. The main difference between these two techniques is that a metabolomic study reflects the "static" state of a metabolome while in a metabonomic study the focus is on the dynamic changes in the metabolome profile due to the applied perturbation to the biological system.

The inspiration for our study comes from the determination of phenolic acids in carrot which, after two months of storage in a fridge, were attacked by unspecified microorganisms. The carrots in question had not been washed after sampling in the field so there were some microorganisms present which after time developed to multiple colonies. These carrots were then washed and phenolic acids were determined. The comparison of the content of phenolic acids with the original unspoilt samples from the same field and plots was expressed in relative numbers as attacked carrot / reference carrot. Reference carrots are carrots analyzed immediately after delivery to the laboratory. Relative numbers are given in Figure 5.

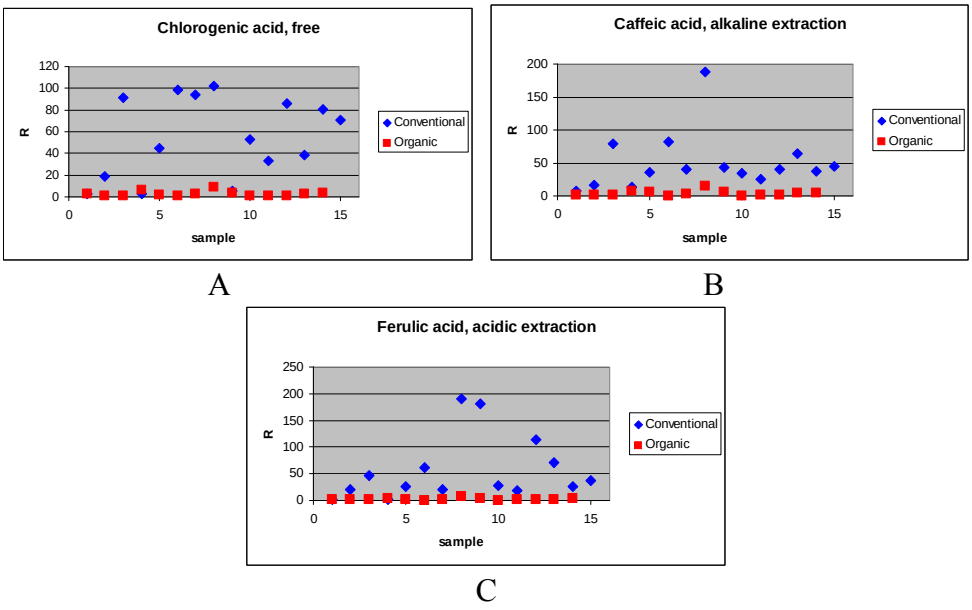


Figure 5. Relative change in the content of certain phenolic acids. (A) free phenolic acids, (B) alkaline and (C) acidic extraction.

In this case 14 samples of organically grown carrot were available. Figures 5 show a remarkable increase of the content of phenolic acids in conventionally grown carrots relative to the organically grown ones. The increase in free chlorogenic acid is more than 60-fold in conventionally grown carrots, while in organically grown carrots the increase is less than 6-fold.

Our aim was:
1. To perform a metabolomic study checking the metabolome "fingerprint" in organically and conventionally grown carrots.
2. To apply controlled microbiological attacks as well as procedures generating a primed state in the plants, evaluate their impact on the phenolic acids content as well as check other metabolome fingerprints.

3. MATERIALS AND METHODS

Carrots were sampled during the 2007 and 2008 harvest seasons. In both years the carrot variety was Nerac.
In 2007 we had the possibility to select between two environments, one close to Eghezée and the other close to Borlez; both cities are located in the Walloon region of Belgium. In each environment we had two fields – one with conventional grown carrot and one with organically grown carrot. The fields within one environment were close together – distance around 5 km. Within each field we selected three parcels – squares of 10 m, the distance between parcels was 15 m. From the corners of each parcel we took the samples. The fifth sample was taken from the middle of the parcel. From each field we collected 15 samples. Each sample was in fact composed of several carrots in order to have enough material for analysis. This is the usual sampling approach followed in the ADAB project.
In 2008 the same sampling approach was adopted except that the locations were different due to the crop rotation. One environment was close to Noville (region of Eghezée) and the second one close to Borlez, both in Belgium. In the region of Eghezée, the distance

between the paired fields was around 9 km while in the region of Borlez the distance was around 1 km.
Based on phytopathological examinations we have selected the environment with the lowest incidence of microbiological contamination. In 2007 and 2008 the environment selected for the pathogenecity test and metabonomic study was Borlez.
Samples from each plot were labelled and packed independently. After delivery to the laboratory defined reference carrots from each plot were immediately washed, cut into slices and lyophilised, after which they were homogenised and stored in a freezer before analysis. Other samples were separated in various batches based on the purpose they were taken for and distributed to partners for analysis.

3.1. Field sampling

3.1.1. Samples for metabolomic study

Samples were collected from both environments in both years. For each parcel a pooled sample was prepared and placed in ice during the transport to the laboratory. This was done by mixing carrots from five sampling spots within the parcel. Once at the laboratory, the carrots were cut, lyophilised and stored at -20 ^{o}C before transfer to Ispra for analysis. It is worth to mention that in case of the 2007 carrot samples, they were harvested in 1 – 3 October and the lyophilised samples were stored in the freezer up to their analysis in Ispra (end of February 2009). In summary, for each of the 2007 and 2008 harvest seasons the metabolomic study was done on 12 lyophilised samples of carrots collected from the fields (i.e. 2 environments x 2 fields x 3 parcels). These 24 carrot samples were labelled as "Fresh".

In addition another set of 12 samples coming from the same environments and fields of 2008 were stored at the manufacturer from their harvest (29/09/2008) up to 22/01/2009. Then these samples were lyophilised and dispatched to Ispra. The storage conditions at the manufacture were a temperature of 1 °C and a relative humidity of 90 % but not monitored. These 12 carrot samples were labelled as "Stored".

Consequently, a total of 36 carrot samples were then produced. Once the samples have been registered in Ispra, they were stored at -80 ^{o}C. The deviation from the usual sampling approach was necessary due to the demand to reduce the amount of samples for the metabolomic study.

3.1.2. Samples in 2007 for pathogenecity and metabonomic tests

- 15 samples – reference measurement (no treatment of the carrots). Part of the samples that was dedicated for metabolomic study.
- 15 samples for BABA priming and dedicated for phenolic acids determination after priming.
- 15 samples for BABA priming and dedicated for pathogenicity tests (lesion size).
- 15 samples for microbiological inoculation - 6 mm wound - followed with phenolic acids determination.
- 15 samples for pathogenicity tests – 6 mm wound (lesion size).

The described batches of samples were equal for organically and conventionally grown carrots, apart from the growing systems.

3.1.3. Samples in 2008 for pathogenecity and metabonomic tests

In addition to the samples taken for the same purposes as in 2007 other samples were collected in 2008:

- 15 samples for chitosan priming and dedicated for phenolic acids determination after priming.
- 15 samples for chitosan priming and dedicated for pathogenicity tests (lesion size).
- 15 samples for microbiological inoculation – 1 mm wound - followed with phenolic acids determination.
- 15 samples for pathogenicity tests – 1 mm wound (lesion size).

In this harvest season additional samples were collected for preliminary experiments with chitosan priming and microorganisms testing. The reasons were to check microorganisms activity and test various procedures for chitosan application.

3.2. Microbiology

3.2.1. Field evaluation

Two visits on the carrot fields were done. During each visit the phytosanitary quality of the crop in each field was assessed by FUSAGx.
In the three parcels per field, the selected plots for sampling were examined. Per sample plot, 6 to7 carrots were observed; symptoms and pathogens were recorded for each carrot. Furthermore, a general observation of the parcel was carried out specifically for Alternariosis symptoms and a percentage of diseased plants were assessed.

3.2.2. Aggressiveness test

Ten strains of *Sclerotinia sclerotiorum* were bought at MUCL (*Mycothèque* of *Université de Louvain-la-Neuve, Belgique*) and CBS (*Centraalbureau voor Schimmel Cultures*, The Netherlands) (Table 1). Each strain was stored at FUSAGx at -80 °C in a 30 % glycerol solution until use. *S. sclerotiorum* cultures were initiated from cryotubes by placing 100 µL of suspension on Potato Dextrose Agar (PDA) at 25 °C for 15 days. A second subculture of 10-day-old colony also grown on PDA medium was used to obtain a layer of mycelium. That layer was pre-cut with a cork borer (6 mm of diameter).

- Before use, the carrots were carefully washed. Carrots were disinfected by soaking for two minutes in sodium hypochlorite solution (10 %) and rinsed twice in sterile distilled water. After drying, carrots were wounded: one wound (6 mm in diameter, 4 mm deep) was created 2 – 3 cm beneath the leaves using a cork borer and the pathogen immediately inoculated.
- Carrots (16 per strain of pathogen) were incubated at 25 °C under saturated atmosphere and dark conditions for seven and nine days before measurement of lesion size (in mm).

Table 1: Characterization of *S. sclerotiorum* strains.

Strains	Origin of isolation (country)	Institute of micro-organisms collection
MUCL1154	*Cichorium intybus*, common chicory (Chile)	*Mycothèque of Université de Louvain-la-Neuve*, Belgium
MUCL8095	Unknown (South Africa)	*Mycothèque of Université de Louvain-la-Neuve*, Belgium
MUCL9083	Seed of Scorzonera sp. (Belgium)	*Mycothèque of Université de Louvain-la-Neuve*, Belgium
MUCL16059	Soil (Scotland)	*Mycothèque of Université de Louvain-la-Neuve*, Belgium
MUCL18183	Stem of *Carthamus tinctorius*, Safflower (Morroco)	*Mycothèque of Université de Louvain-la-Neuve*, Belgium
MUCL30163	Unknown (Unknown)	*Mycothèque of Université de Louvain-la-Neuve*, Belgium
CBS537.77	Flowers of *Helianthus annuus*, Sunflower (The Netherlands)	*Centraalbureau voor Schimmel Cultures, The Netherlands*
CBS499.50	*Linum usitatissimum*, common flax or linseed (The Netherlands)	*Centraalbureau voor Schimmel Cultures, The Netherlands*
CBS344.58	*Medicago sativa*, alfalfa (The Netherlands)	*Centraalbureau voor Schimmel Cultures, The Netherlands*
CBS397.54	*Matthiola* sp. (Unknown)	*Centraalbureau voor Schimmel Cultures, The Netherlands*

3.2.3. Pathogenicity test

The strain of *S. sclerotiorum* (strain MUCL 30163) was prepared at FUSAGx as mentioned in 3.2.2. aggressiveness test.

Carrots conventionally grown were thoroughly washed and stored at 1 °C and 90 % RH. Carrots were disinfected by soaking for two minutes in sodium hypochlorite solution (10 %) and rinsed twice in sterile distilled water. After drying, carrots were prepared as followed:

- One wound per carrot (6 mm in diameter, 4 mm deep) was created 2 – 3 cm beneath the leaves using a cork borer. The pathogen was cut with a cork borer of same size and immediately inoculated;
- One wound per carrot (1 mm in diameter, 4 mm deep) was created 2 – 3 cm beneath the leaves using a cork borer. The pathogen was cut with a cork borer of same size and immediately inoculated;
- The pathogen was immediately inoculated to the carrot without any wound.

Carrots (15 per type of treatment) were incubated at 25 °C under saturated atmosphere and dark conditions for three and seven days before measurement of lesion size (in mm).
The strain of *S. sclerotiorum* was prepared as mentioned in 3.2.2. Aggressiveness test and carrots were also prepared and inoculated as already described. Two batches of carrots were used per field (organic or conventional). The first batches were sent to IRMM for HPLC analysis whereas the second batches were kept for incubation for five and eight days at 25 °C. Finally, additional sets of carrots were artificially primed with BABA at IRMM, then FUSAGx inoculated and incubated these carrots for analyse of tissue resistance.
In the 2008 harvest season BABA and chitosan were used for priming at IRMM. Samples primed with chitosan were handled the same way as samples primed with BABA. In addition another way of inoculation was adopted (i.e. 1 mm wounding).
Data were subjected to statistical analysis with ANOVA using SAS. The treatment means were separated at the 5 % significance level using the Newman and Keuls test.

3.3. Phenolic acids determination

3.3.1. Extraction

Each sample set dedicated for phenolic acids determination was lyophilised (Christ, Delta 1-24 LSC, Osterode, Germany) and homogenized (Retsch, ZM 200, Haan, Germany). Samples were stored in the freezer (-18 ^{o}C) before analysis. Water content was determined in each sample (Metrohm, 701 KF Titrino or 774 oven sample processor, Herisau, Switzerland) before extraction of phenolic acids.

Three independent **extraction procedures** were applied

1. Free phenolic acids determination. One gram of the sample was weighed (Mettler Toledo, PB-303, Kusnacht, Switzerland) to three digits in an amber glass vial. Extraction mixture was methanol/water (v/v) 80/20 – 10 mL, process was accelerated with ultrasonic bath (VWR, Branson 5510, Brussels, Belgium) for 30 minutes. Afterwards mixture was filtrated and filtrate collected in amber glass vial ready for analysis with HPLC.
2. Acidic extraction. One gram of the sample was weighed in amber glass vial. Extraction mixture was methanol/water (v/v) 80/20 – 8 mL and 2 mL of 30 % solution of HCl. Extraction proceeded overnight. Afterwards mixture was filtrated and filtrate collected in amber glass vial ready for analysis with HPLC.
3. Alkaline extraction. One gram of the sample was weighed in amber glass vial. Extraction mixture was methanol/water (v/v) 80/20 – 8 mL and 2 mL of 30 % solution of NaOH. Extraction proceeded overnight. Afterwards mixture was acidified with HCl to pH = 6 and filtrated. Filtrate collected in amber glass vial was ready for analysis with HPLC.

3.3.2. High Performance Liquid Chromatography (HPLC) determination

Twelve phenolic acids were selected - gallic, 3,4-dihydroxybenzoic, 4-hydroxybenzoic, vanillic, caffeic, syringic, *p*-coumaric, ferulic, sinapic, salicylic (all from Fluka, Buchs, Switzerland), trans-cinnamic (from Aldrich, Steinheim, Germany) and chlorogenic (from Sigma, St. Louis, USA).
Phenolic acids were quantified with HPLC Bio-Tek (Milan, Italy). Samples were injected with Autosampler 560 cooled with external thermostatic bath at 5 ^{o}C. A Diode Array Detector 540 was used for quantification at 280 nm wavelength for all phenolic acids. Tertiary pump Kontron Instruments 525 (Milan, Italy) was used for gradient separation. Zorbax SB-C18 (Agilent Technologies, Palo Alto, USA) 4.6 x 250 mm with 5 μm particles was used for separation. A binary gradient (57 minutes) starting from 100 % acidified water (0.03% solution of phosphoric acid) and finished with 100 % methanol was sufficient for suitable separation. The same gradient was used for all extraction methods. External standard procedure was used for quantification. A chromatogram of a mixed standard of phenolic acids is shown in Figure 6.
This fit for purpose procedure was already used in the main ADAB project for phenolic acids determination. Selected performance parameters of the method are given in Table 2.

Table 2: Selected performance parameters for the determination of phenolic acids in carrot.

Phenolic acid	Recovery (%)	RSD (%)	LOD (mg/kg)
Gallic	90	5	2
4-H. benzoic	96	4	5
Chlorogenic	83	12	13
Caffeic	91	1	6
Syringic	82	18	8
Ferulic	92	1	3
Sinapic	112	6	2

3.4. Metabolomic measurements

Metabolomic measurements were performed at JRC, IHCP, Ispra, Italy. They applied their own procedure for samples handling and registration. This was due to the different amount of samples dedicated for metabolomic study.

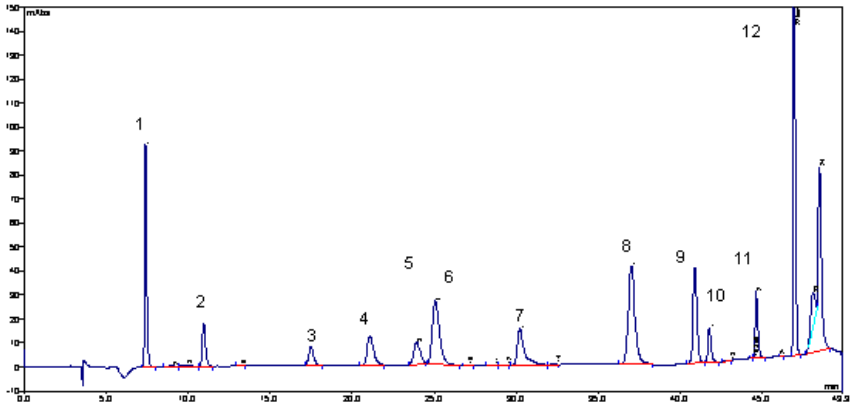


Figure 6. Chromatogram of phenolic acid standards.
1 - gallic acid, 2 – 3,4-dihydroxybenzoic acid, 3 – 4-hydroxybenzoic acid, 4 – chlorogenic acid, 5 – vanilic acid, 6 - caffeic acid, 7 – syringic acid, 8 – *p*-coumaric acid, 9 – ferulic acid, 10 – sinapic acid, 11 – salicylic acid, 12 – trans-cinnamic acid.

3.4.1. Sample Registration

Carrot samples (n = 36) were registered using the software EuroDat 4.0. Different information including agricultural system used (conventional versus organic), location (environment 1 and 2), collection year (2007 and 2008), and lyophilised samples from "fresh" and "stored" carrots, were registered for each sample and recorded in the document “EuroDat Carrot Samples.xls”. An eight digit number code characterized each single carrot sample (see annex 1).

3.4.2. Extraction

For the ^{1}H NMR analysis, 50 ± 2 mg of each sample were weighed in 1.5 mL Eppendorf cups and extracted for 32 min in an ultrasonic water bath with 0.5 mL of CD_3OD for each samples. Three blanks were also prepared as well as six replicates (A – F) of each one of three different samples (09010013; 09010120; 09010129). A total of 57 (36 samples + 3 blanks + 18 replicates = 57) samples were produced and then centrifuged (15 rcf, 5 min, 12 ^oC) and the liquid phases were used directly.
For the MS, the same procedure was followed using in this case CH_3OH instead of deuterated methanol as solvent for the extraction and preparing 2 blanks, 8 pure methanol samples, 5 replicates (a-e) of two different samples (09010013 and 09010116 although sample 09010116c was lost), 1 mixture of standards (Sulphadimethoxine, Val-Try-Val, Leucine–Enkephalin, Eritromicine in 2% CH_3CN - injected 6 times for each analysis), 1 mixture of organic/environment 1 samples, 1 mixture of organic/environment 2 samples, 1 mixture of conventional/environment 1 samples, 1 mixture of conventional/environment 2 samples, and 1 mixtures of these carrot sample mixtures used as control (injected 6 times for each analysis), for a total number of 62 samples and 71 injections for each analysis. All the mixtures of carrots used as a control were made by mixing samples from both years.

3.4.3. ^{1}H NMR analysis

^{1}H NMR spectra were registered on a Bruker (Rheinstetten, Germany) DRX-500 instrument operating at 500.13 MHz for ^{1}H observations using a Broadband Inverse (BBI) microprobe maintained at 298 K. ^{1}H NMR spectra were digitized into 64K data points over a spectral width of 14 ppm with an acquisition time of 3.5 s. An additional relaxation delay of 3.5 s was included, making a total recycling time of 7.0 s. A 90° pulse was used with 64 scans. Spectra were Fourier transformed applying a line broadening apodization function of 0.3 Hz. NMR spectra were processed in Bruker TopSpin 1.3. Chemical shifts are referenced to the solvent residual peak (CHD_2OD, 3.1 ppm). All spectra were manually phased and baseline corrected. Spectra were integrated using the AMIX 3.8.6 software (Bruker) into a series of 242 bins (buckets) of 0.04 ppm over the range of chemical shifts from 10 ppm to 0 ppm. Spectral region from 5.04 - 4.72 ppm was removed from the integration procedure because of the presence of residual water signal. The intensity of the buckets was normalized to the total intensity of the spectrum. These buckets represent the descriptors for multivariate analysis.

3.4.4. UPLC conditions

Separations were carried out on an Acquity UPLC system (Waters, Milford, MA, USA) with a Waters Acquity UPLC column (HSS T3, 2.1 mm x 50 mm, 1.8 μm) fitted with a Waters Acquity Van Guard precolumn (HSS T3, 2.1 mm x 5 mm, 1.8 μm). The injection volume was fixed at 2 μL in the partial loop mode. Samples were stored at 4 $^\circ$C in the autosampler prior to analysis. The column oven temperature was maintained at 60 $^\circ$C and the flow rate was 0.6 mL/min. Solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in methanol. The gradient profile for the mobile phase was as follows: t (0-1), 5% B; t (1-2.63), 61% B; t (2.64-5.61), 76% B; t (5.62-7.98), 95% B; t (7.99-8.50) 95% B; t (8.51-10.00), 100% B; t (10.01-12), 5% B (where t refers to time in min).

3.4.5. QTOF/MS parameters

Analyses were performed using a Micromass-Q-Tof Premier mass spectrometer – QTOF/MS (Waters) equipped with an ElectroSpray Ionization (ESI) source. Four runs were performed for each sample in positive (triplicate) and in negative (one) ESI mode. MS operating conditions were set as follows: the desolvation gas flow was 400 L/h at a temperature of 200 °C, the capillary voltages were defined as 3.0 kV in positive and 2.4 kV in negative ion mode. In both modes, the cone voltage was constant at 30 V, the micro-channel plates (MCPs) was at 1700 V, the source temperature was 100 °C, the cone gas flow was set to 35 L/h, and the collision gas flow was 0.30 mL/min. The QTOF/MS system was operated in wide-pass quadrupole mode with a low and high collision energy (2 eV and a ramp from 15 to 45 eV respectively) to acquire MS information. Data were collected in W-optics centroid mode over a m/z range of 50–1000 with a scan time of 0.25 s and an interscan delay of 0.02 s. For the Lockmass, a solution of leucine–enkephalin at 0.1 ng/mL (Sigma–Aldrich, Buchs, Switzerland) was infused through the Lock Spray probe at 20 μL/min. Prior to the sample analysis, the calibration of the ESI-QTOF-MS detector in both ion modes was achieved using a solution of 0.5 % formic acid in 9:1 2-propanol:water with 0.05 M sodium hydroxide. Data acquisition, data handling and instrument control were performed using MassLynx Software (Waters).

3.5. Metabonomic measurements

For metabolome extraction all three procedures used for phenolic acids determination were applied. Extracted compounds were detected with the same procedure applied for phenolic acids determination. We have determined phenolic acids and content of unknown compounds were estimated as standardized amount - area of their peak relative to the sample used (mAbs*min/sample dilution). Sample dilution reflects amount of sample (dry powder) and extraction solvent.
Results were also evaluated based on the relative numbers as described in the Introduction.
We have focused only on those unknown compound indentified in our metabolome profile which offers potential for discrimination organically and conventionally grown carrots.

4. RESULTS AND DISCUSSIONS

4.1. Microbiology tests

4.1.1. Field evaluation results – 2007 harvest season

The results of the first field evaluation are presented in Tables 3 and 4, for conventional and organic fields respectively. Whatever the field, a considerable presence of slugs was recorded (26.6 % to 28.3 % of incidence). Alternariose was present in both fields, but particularly in the organic one. *Sclerotinia sclerotiorum* was observed only in the organic field (4.3 % of incidence). Finally, within the same field, differences could be observed from one parcel to another.

Table 3: Incidence of diseases (%) on carrots observed in the conventional field (region of Eghezée) in 2007.

	Number of carrots	Slugs	Black spots after slugs	*Sclerotinia sclerotiorum*	Alternariose
Parcel 1	34	14.70	11.76	0.00	0.10
Parcel 2	30	26.67	10.00	0.00	0.05
Parcel 3	30	40.00	0.00	0.00	0.05
TOTAL	94	26.60	7.45	0.00	0.07

Table 4: Incidence of diseases (%) on carrots observed in the organic field (region of Eghezée) in 2007.

	Number of carrots	Slugs	Black spots after slugs	*Sclerotinia sclerotiorum*	Alternariose
Parcel 1	31	32.26	19.35	12.90	10.00
Parcel 2	30	33.33	13.33	0.00	5.00
Parcel 3	31	19.35	0.00	0.00	5.00
TOTAL	92	28.26	10.87	4.35	6.67

In the second environment (i.e. region of Borlez) the field evaluation results are in Table 5. In this case 54 samples were collected from each field and evaluated.

Table 5: Incidence of diseases (%) on conventionally and organically grown carrots in environment 2 (region of Borlez) in 2007.

Field	Number of carrots	Slugs	Black spots after slugs	*Sclerotinia sclerotiorum*	Alternariose
Conventional	54	9,2	5.6	0.0	-
Organic	54	22,2	5.6	1.8	-
TOTAL	108	15.7	5.6	0.9	-

It was decided to use carrot samples grown in environment 2 as the incidence of diseases was lower than the one observed in environment 1. Indeed, it is expected that a priming effect should more likely occurred in case of lower biotic stress.

4.1.2. Field evaluation results – 2008 harvest season

In this year the same methodology for evaluation were adopted as in 2007 harvest season.

Table 6: Incidence of diseases (%) on conventionally grown carrots (region of Eghezée) in 2008.

	Number of carrots	Slugs	Caterpillar	Gale (Streptomyces)	Alternariose	Powdery mildew
Parcel 1	25	0	0	5	9	0
Parcel 2	25	0	0	1	9	0
Parcel 3	25	0	0	2	7	0

TOTAL	75	0	0	8	25	0
Incidence (%)		0	0	11	33	0

Table 7: Incidence of diseases on organically grown carrots (region of Eghezée) in 2008.

	Number of carrots	Slugs	Caterpillar	Gale (Streptomyces)	Alternariose	Powdery mildew
Parcel 1	25	0	0	0	5	15
Parcel 2	25	0	0	0	4	11
Parcel 3	25	0	0	0	7	12
TOTAL	75	0	0	0	16	38
Incidence (%)		0	0	0	21	51

Table 8: Incidence of diseases (%) on conventionally grown carrots (region of Borlez) in 2008.

	Number of carrots	Caterpillar	Root Aphid	Alternariose	Click beetle
Parcel 1	25	1	5	9	0
Parcel 2	25	6	3	1	0
Parcel 3	25	2	0	3	0
TOTAL	75	9	8	13	0
Incidence (%)		12	11	17	0

Table 9: Incidence of diseases on organically grown carrots (region of Borlez) in 2008.

	Number of carrots	Caterpillar	Root Aphid	Alternariose	Click beetle
Parcel 1	25	0	4	6	0
Parcel 2	25	0	0	7	0
Parcel 3	25	2	0	7	1
TOTAL	75	2	4	20	1
Incidence (%)		3	5	27	1

In the 2008 harvest season the fields in the region of Borlez were selected for experimental trial.

4.1.3. Strain selection – aggressiveness test

All selected strains were able to infect carrots. However, strains MUCL16059 and CBS344.58 did not present regular symptoms after respectively seven and nine days of incubation. The highest aggressiveness was observed for MUCL9083 and CBS397.54, but these strains also presented a high standard deviation. Finally, strain MUCL30163 was selected for further experiments because it allowed a relatively high level of aggressiveness together with a low value of standard deviation (lowest coefficient of variation of 12 % and 15 %, after seven and nine days of incubation, respectively).
It is well known that a certain variability is to be expected when reproducing the symptoms of a disease. Here, as the protocol was standardized, it is thought to be mainly due to the strain factor.

4.1.4. Pathogenesis tests

4.1.4.1.Harvest season – 2007

The length of lesion was measured after five and eight days from inoculation.
No statistical difference was observed between organically and conventionally grown carrots whether they were primed or not.
The protocol of symptoms repeatability (variation of lesion size) developed in part 2 revealed a relatively high level of aggressiveness together with a low value of standard deviation. That high level might induce a systemic resistance. In order to obtain priming of ISR (induced systemic resistance), a protocol with lower level of infection must be developed.

4.1.4.2.Harvest season – 2008

The same strain was used for pathogenesis study (strain MUCL 30163). The test with fresh carrots offered similar results as with carrots collected in the 2007 harvest season. There was no observed difference in lesion size after inoculation in organic or conventional carrots. Results with BABA primed carrot are summarised in Figures 7 and 8.
An ANOVA shows significant difference between organically and conventionally grown carrots. After five days in case of the 0.1 cm inoculation the probability factor is $p = 0.003$, while for the 0.6 cm inoculation $p = 0.01$. After 7 days of incubation in case of the 0.1 cm inoculation $p = 0.004$ and for the 0.6 cm of inoculation $p = 0.04$.

4.2. Metabolomic study

4.2.1. Strategy for the extraction and analysis

As explained in Material and Methods, 0.5 mL of methanol were added to 50 ± 2 mg of each carrot sample and then extracted for around 30 min in the ultrasonic water bath; after centrifugation, the liquid phase of each sample was directly used for metabolic fingerprinting analysis.

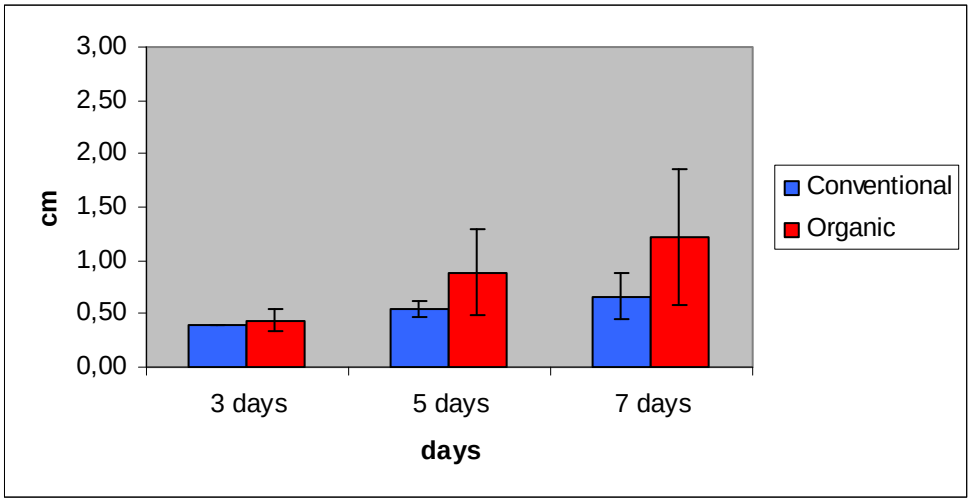


Figure 7. Lesion size after 0.1 cm wound inoculation.

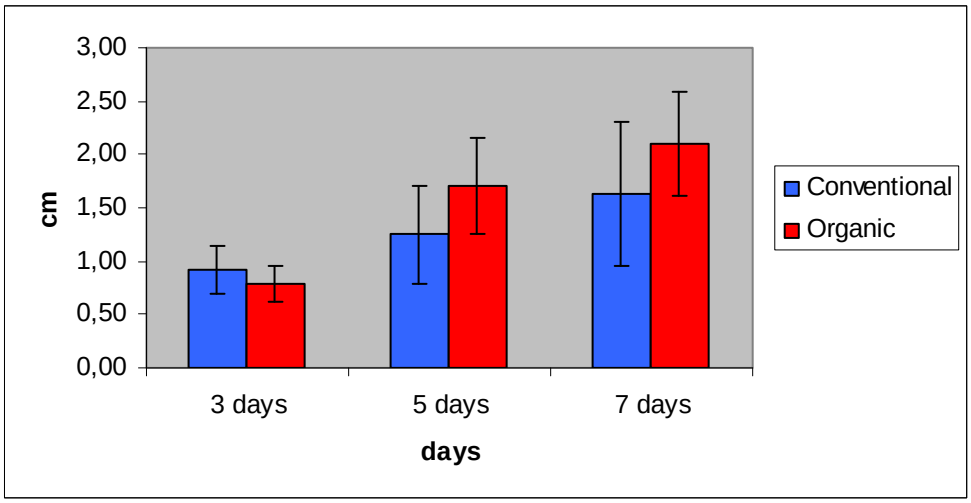


Figure 8. Lesion size after 0.6 cm wound inoculation.

The choice of the solvent used for the metabolite extraction is critical. This step can strongly affect the final result of any metabolomic study where a solid material needs to be extracted with a liquid phase before chemical analysis. The general approach in natural product chemistry consists of consecutive and exhaustive extractions with solvents of increasing polarity (i.e. hexane-chloroform-methanol) (Heinrich et al. 2004). This method produces different raw extracts where the compounds are roughly separated on the basis of their polarity. Unfortunately, this kind of extraction methods has not been automated yet and current metabolomic analysis relies on the analytical data obtained from the analysis of a single type of extract (Choi and al. 2004; Xie and al. 2008; Okada and al. 2009). Using a specific solvent (or mixture of solvents) will produce a target extract where only certain classes of compounds will be present, and this fact is in contrast with any truly metabolomic analysis. However, a technology that would allow an exhaustive metabolic extraction and at the same time be able to be coupled with the spectroscopes does not exist yet, so scientists need to decide *a priori* the specific solvent system to be used for the extraction. In our case 100% methanol was chosen with the aim to obtain an extract having a wide range of chemical diversity and, at the same time, being directly usable for the analysis with two analytical methods, Nuclear Magnetic Resonance (NMR) spectroscopy and Ultra-performance Liquid Chromatography

Quadrupole Time of Flight Mass Spectrometry (UPLC-QTOF/MS). Mixture of solvents (such as methanol/waters for instance) are not easily analyzable directly by NMR, while certain other solvents such as chloroform can disturb or even damage the MS detector. In our case the extraction was repeated two times, the first with deuterated methanol (for NMR analysis) and the second with normal methanol (for LC-MS analysis). This was due to internal reasons of instruments management only, while one single extraction event (with deuterated methanol) can be valid and used for both detectors.
Finally, as mentioned above, the fact that a detector able to supply comprehensive qualitative/quantitative data on all the chemical entities of a crude natural product mixture does not exist yet is also in contrast with any truly metabolomic analysis. The simultaneous use of different detectors is recommended and represents currently the best option; at present, between several available detectors, NMR and MS represent surely the most valuable ones (Dunn, Bailey et al. 2005).

4.2.2. NMR datasets

Apart from the 36 carrots samples, replicates and blanks were also produced and analyzed in order to monitor and validate the overall dataset (total of 57 samples). Samples 09010118 (organic, region of Eghezée) and 09010122 (conventional, region of Borlez), for instance, were extracted five times and the corresponding extracts were analyzed by ^{1}H NMR. Figure 9 shows the five replicates (a-e) of the first sample from which can be visually observed the reproducibility of the extraction procedures in both, qualitative (signal chemical shifts) and quantitative (signal intensities) aspects.

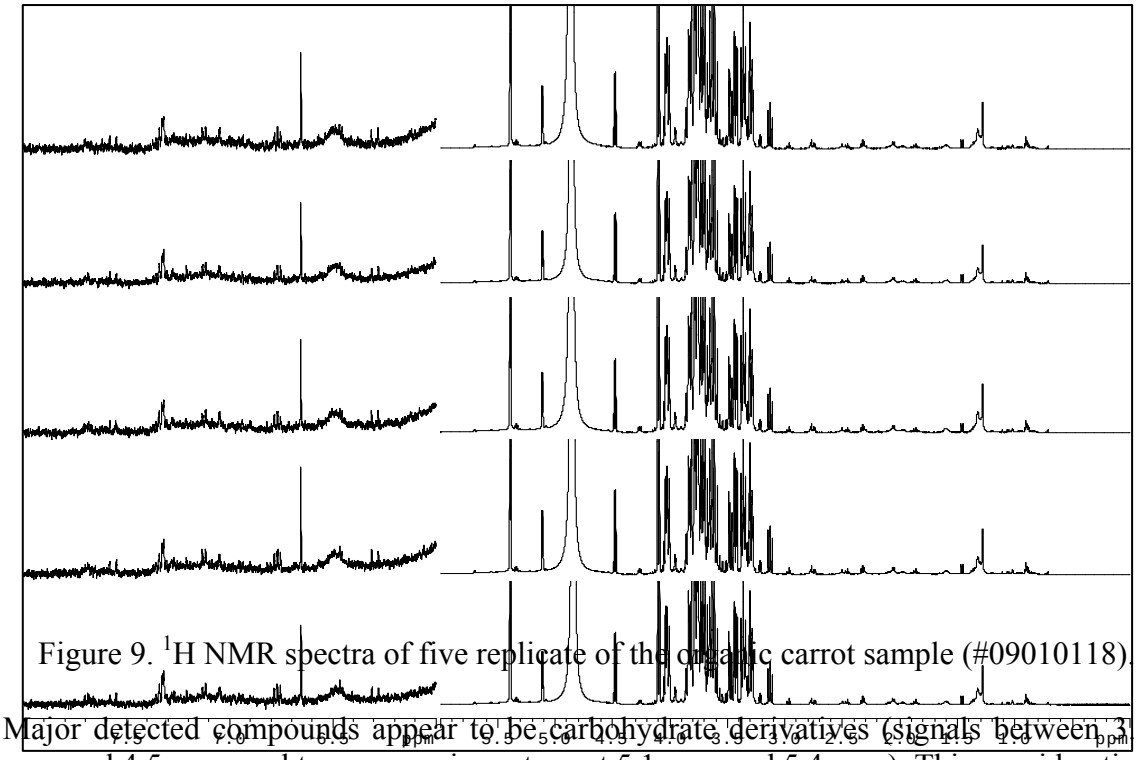


Figure 9. ^{1}H NMR spectra of five replicate of the organic carrot sample (#09010118).

Major detected compounds appear to be carbohydrate derivatives (signals between 3.1 ppm and 4.5 ppm, and two anomeric protons at 5.1 ppm and 5.4 ppm). This consideration was supported by the fact that few days after the preparation of the extracts, white crystals start to precipitate in the NMR tubes and in the Eppendorf cups; this can be due to the instability of pure sugars in methanol solution.
Visual comparison of certain NMR regions of the proton spectra of sample 09010118 and 09010122 (Figure 10) indicates the presence of some minor chemical differences between both samples (circled in red). These two samples are part of the set of carrots that were stored at the manufacturer during 4 months before analysis (see Material and Methods).

Principal Component Analysis (PCA) applied on the five replicates of both samples (Figure 11) allowed the separation along the PC1 (42.62%) of both samples, while the internal variance indicating the reproducibility of the extraction procedures observed along PC2 appear to be less relevant.

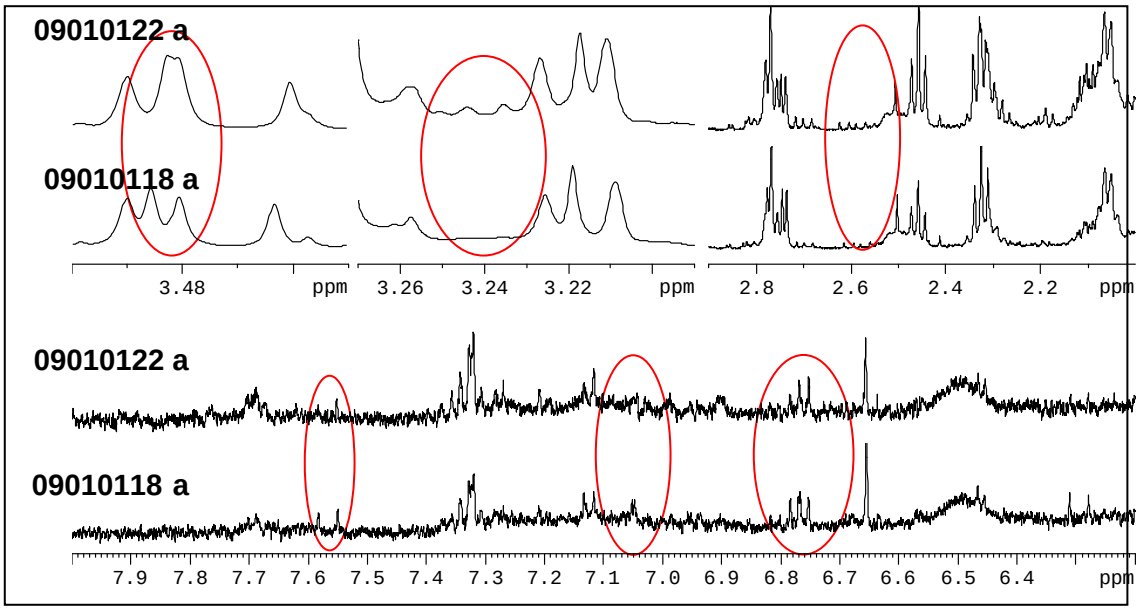
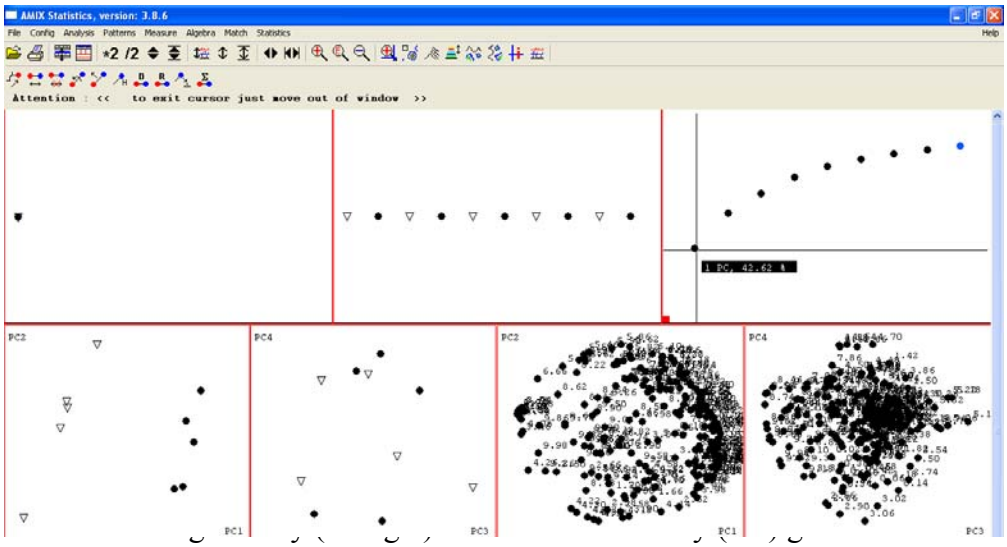


Figure 10. Visual comparison of selected part of ^{1}H NMR showing minor differences (red circle) between an organically (09010118) and a conventionally (09010122) grown carrots in 2008.



2008.

This preliminary result indicated a certain degree of robustness of the extraction method and the analytical measurement; this approach was therefore applied on the 36 carrot samples.

Some of the proton spectra of the samples belonging to the group of 2008 lyophilised "Fresh" carrots (see Material and Methods) present a much stronger water signal with respect to all the others spectra from the "Stored" group (Figure 12). The higher presence of water in some of the "Fresh" samples could be due to the specific handling protocol applied for this group or to the incorrect timing during the lyophilization process. The strong intensity of the water signal in these spectra affects the intensity and shape of all the rest of the signal in the spectrum. These spectra can not be compared to the others

and the group of 2008 "Fresh" carrots (samples from #09010126 up to #09010137 in annex 1) was therefore excluded from the successive chemometric analysis.

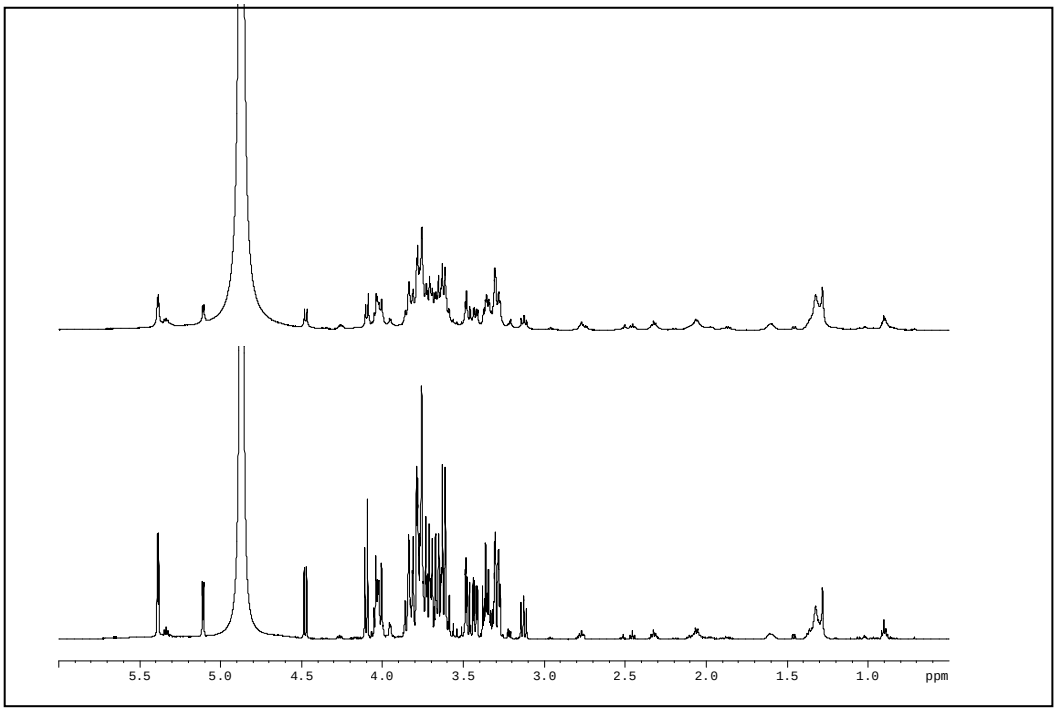


Figure 12. ^{1}H NMR spectra of water determined in 2008 carrot samples lyophilised directly after their sampling.

A PCA was applied to discriminate between individual sub-groups (Organic and Environment 1 versus Organic and Environment 2 versus Conventional and Environment 1 versus Conventional and Environment 2), only in some cases a certain degree of grouping was observed as shown in Figures 14 and 15.

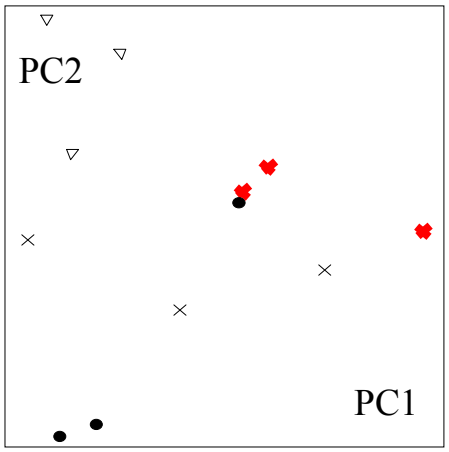


Figure 14. PCA evaluation of results from 2007 harvest season for both environments, conventionally and organically grown carrots.

Org. Env 1; Conv. Env 2; Org. Env 2; Conv. Env 1.

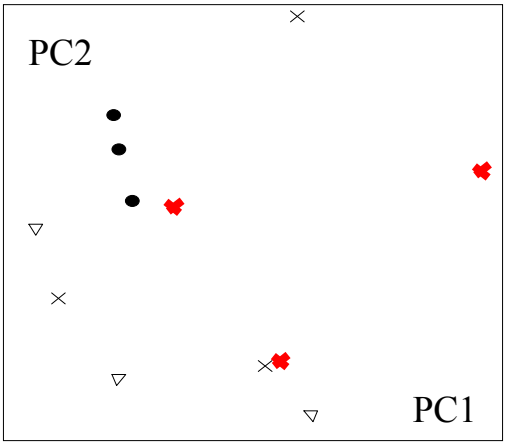


Figure 15. PCA evaluation of results from 2008 harvest season for both environments, conventionally and organically grown carrots.

Org. Env 1; Org. Env 2; Conv. Env 2; Conv. Env 1

It seems that the storage of raw materials is influencing the metabolome; erasing somehow the potential differences observed with fresh lyophilised carrots (fig. 14) even if the powder was stored for a long period in a freezer (more than 1 year).

4.2.3. UPLC-QTOF/MS dataset

The total number of samples analyzed in this case was 66 including the 36 carrot samples, blanks, replicates, mixture of standards, and mixtures of samples; UPLC separation was optimized as well as the QTOF/MS detection that was acquired in both, positive and negative ion modes (see Material and Methods). Figure 16 shows two typical chromatograms obtained in positive ion mode for two carrot samples, one from organic agricultural system in environment 1 (#09010013) compared with the second from conventional agricultural system in environment 2 (#09010120). Visual comparison of both chromatograms highlighted several minor differences in peak detection between both samples (circled in red). Extraction of the corresponding MS spectra of major peaks at retention time (Rt) 1.03 min, 6.28 min, and 8.99 min indicate the great variety, at least in term of molecular weight, of the metabolites contained in these extracts. Moreover, pure sugars do not ionized with the detection condition used here. Therefore, these MS metabolite profiles should be able to highlight different classes of natural products with respect to those one (mainly sugars) observed with the NMR method. This example remarks the complementary characteristics of both NMR and MS detectors and the relevance of their simultaneous use for such kind of untargeted metabolite analysis.
Data extraction and processing (chemometrics) are two tasks currently under consideration.

4.3. Metabonomics

4.3.1. Chemical stress

4.3.1.1. BABA stress, 2007 harvest season

In this study selected phenolic acids were determined and some unknown compounds evaluated from the point of view of discriminating organically and conventionally grown carrots. The evaluation was based on reference numbers defined in Material and methods. Phenolic acids did not offered better discrimination power as shown in Figures 17 A, B, C.

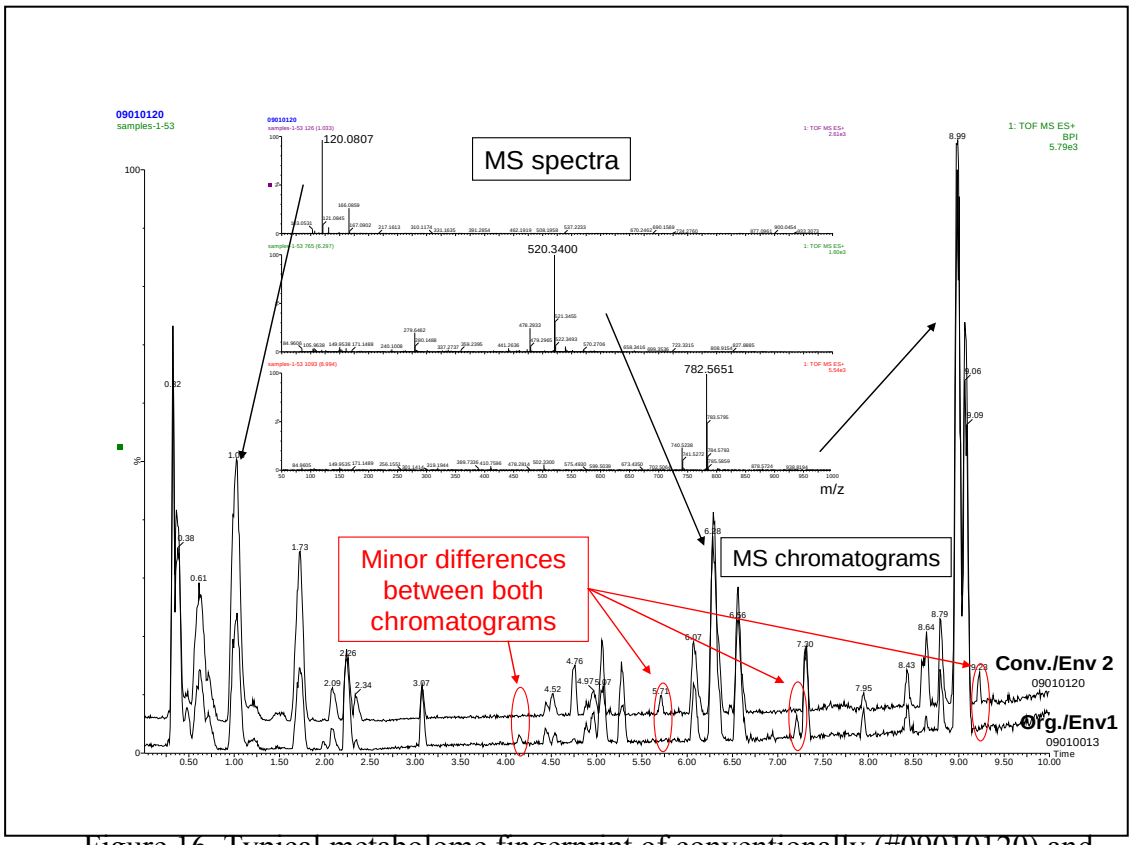


Figure 16. Typical metabolome fingerprint of conventionally (#09010120) and organically (#09010013) grown carrots.

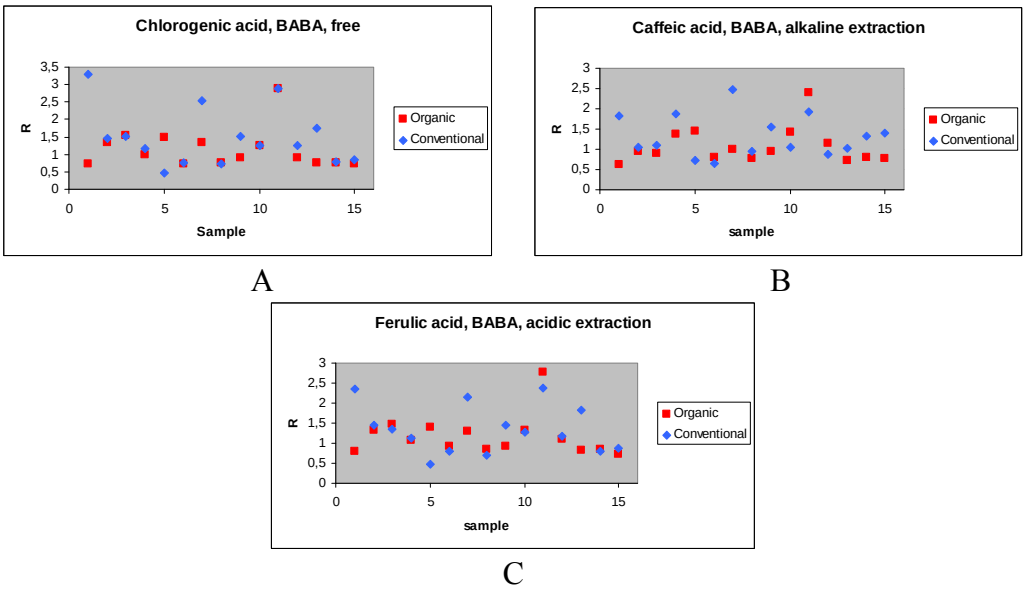


Figure 17. Relative change in phenolic acids content determined after free (A), alkaline (B) and acidic extraction (C). Carrot from 2007 harvest season and primed with BABA.

In all three cases the change in phenolic acids content is highly variable. The BABA stress has no positive effect on discrimination of organically grown carrot from conventionally one.

After evaluation of our fingerprints from all extraction procedures we observed an unknown compound at retention time RT = 8.6 min in the acidic extract (Figure 18). A

comparison of the standardized content of this compound (peak area/sample amount) offers certain uniformity in distribution of the relative numbers (Figure 19).

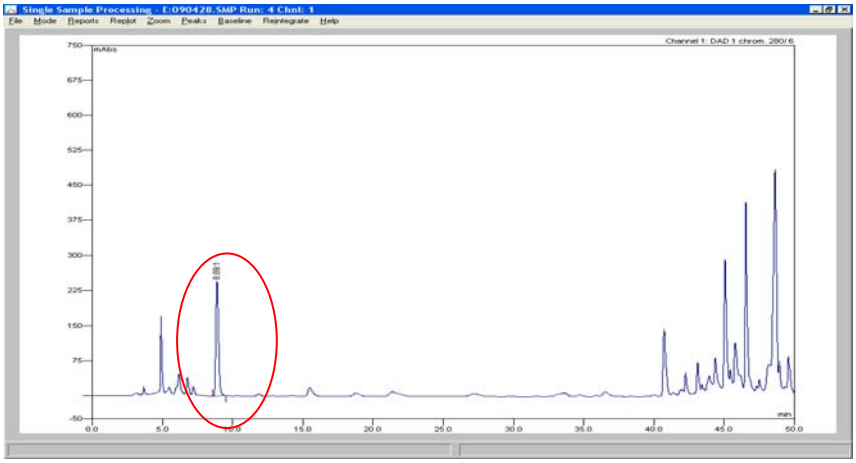


Figure 18. Fingerprint of compounds identified with DAD after acidic extraction of carrot with highlighted unknown compound at RT = 8.6 min.

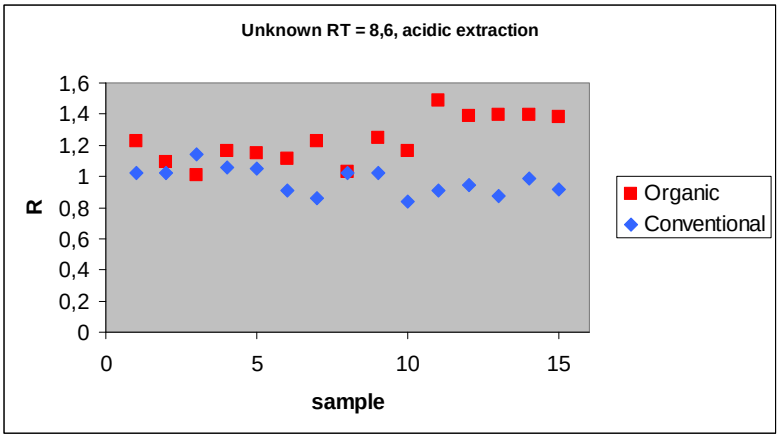


Figure 19. Relative change in the content of unknown compound (RT = 8.6 min) after BABA application for stress and acidic extraction applied for extraction on carrots.

The content of the unknown compound RT = 8.6 min is almost invariable relative to BABA applied stress in organically grown carrot while in conventionally grown carrot there is evidently a slight increase of its content. ANOVA single factor analysis offers $p \leq 0.001$ when comparing both groups of data. It means there is a very highly significant difference between groups of organically and conventionally grown carrots.

4.3.1.2. BABA 2008 harvest season

For these carrots we have applied a new protocol for stress. The concentration of BABA solution was higher in comparison to the one applied in the 2007 harvest season. This change in the BABA priming protocol was reflected also in the change of the content of phenolic acids.

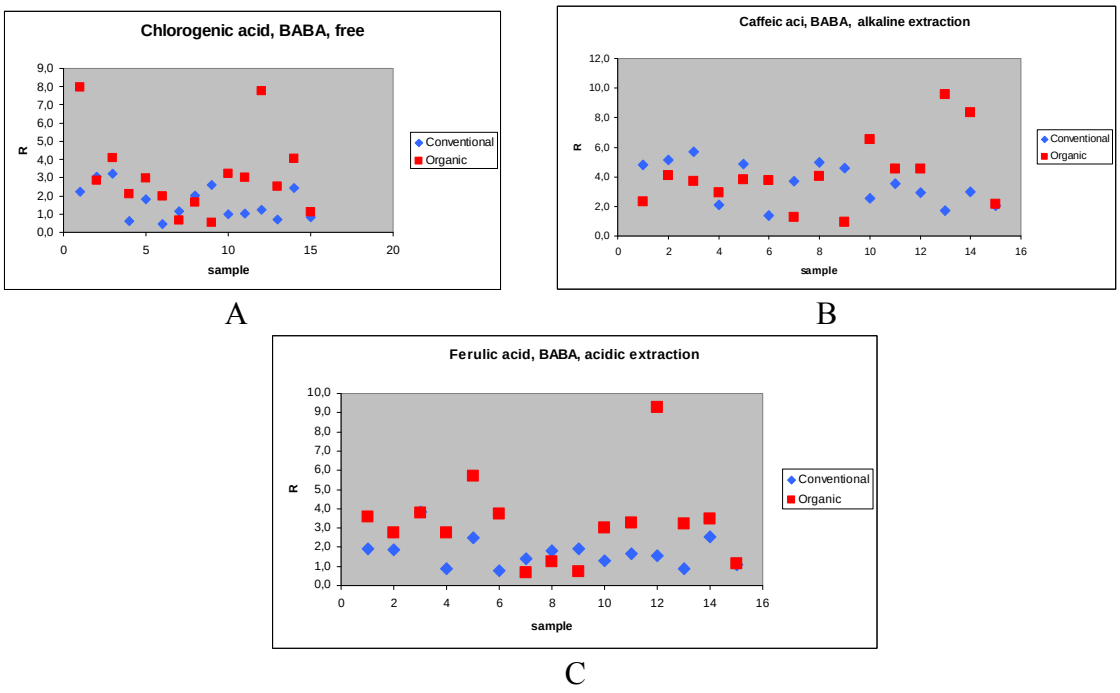


Figure 20. Relative change in phenolic acids content determined after free (A), alkaline (B) and acidic extraction (C). Carrot from 2008 harvest season primed with BABA, new priming protocol.

The results demonstrate an increase in contents of selected phenolic acids determined after three independent extraction procedures (Figure 20). This increase is higher in comparison to those observed with the 2007 BABA priming protocol. An increased level of phenolic acids was probably the reason why we were able to observe a change in lesion size between organically and conventionally grown carrots when the new BABA priming protocol was used in 2008.

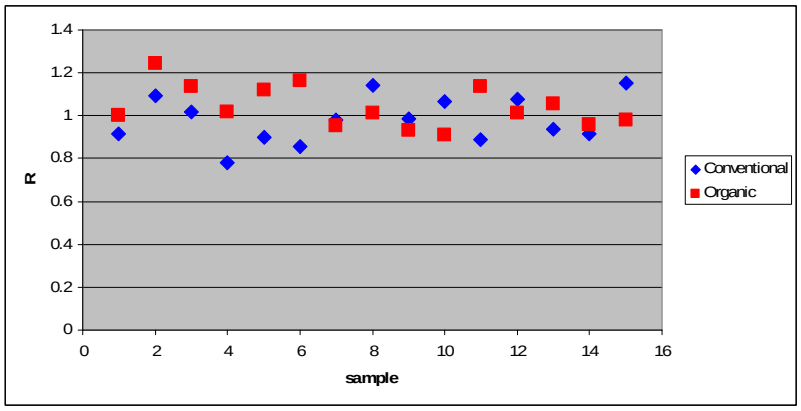


Figure 21. Relative change in content of unknown compound (RT = 8.6) in carrots from both agricultural systems determined after acidic extraction. New BABA protocol applied in 2008 harvest season.

Despite an increase in the content of some phenolic acids, the difference between relative numbers calculated from organically and conventionally grown carrots was not significant ($p = 0.115$). This implicates that BABA has a minor impact on the content of the unknown compound RT = 8.6 min (Figure 21). There is a certain influence on it but it seems that the microenvironment of the carrot is more important than the BABA priming

effect on this compound. During phytopathological evaluation of the fields we observed a higher incident of microbiological attack in 2007 in comparison to 2008 (Tables 5, 8 and 9).

4.3.2. Chitosan priming

The chitosan priming procedure was applied in the 2008 harvest season only.

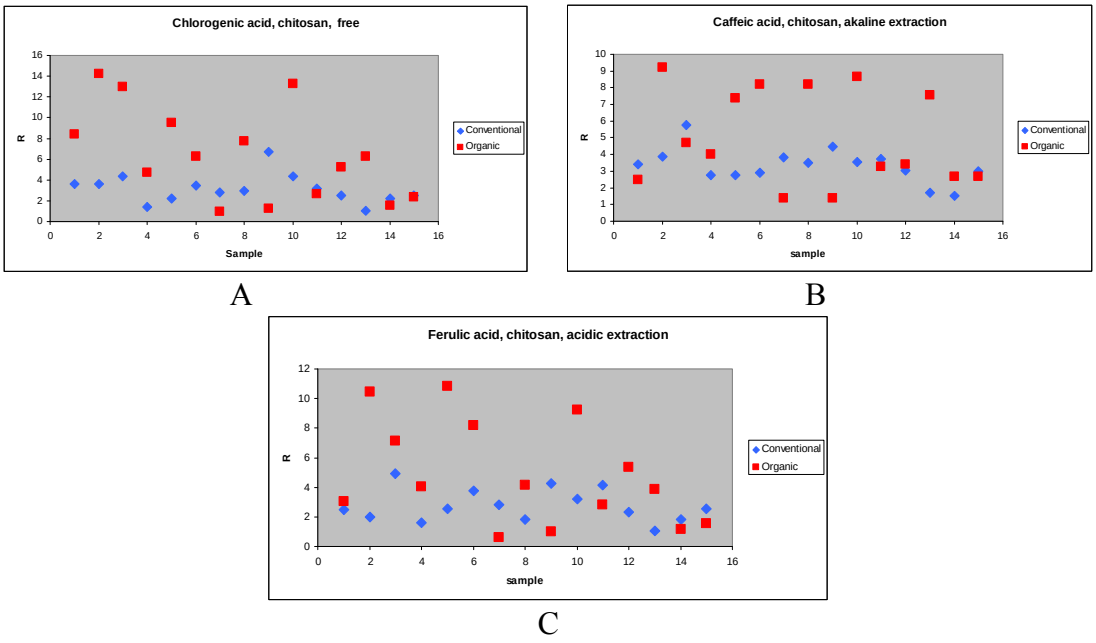


Figure 22. Relative change in phenolic acids content determined after free (A), alkaline (B) and acidic extraction (C). Carrots from 2008 harvest season primed with chitosan.

Chitosan had a more pronounced effect on the content of phenolic acids in carrot than BABA priming (Figure 22). In all cases (applied extraction protocols) the increase was remarkable for both agricultural systems. However, none of the phenolic acid offered discrimination between both agricultural systems. The effect of chitosan priming on the unknown compound (RT = 8.6 min) is reported in Figure 23.

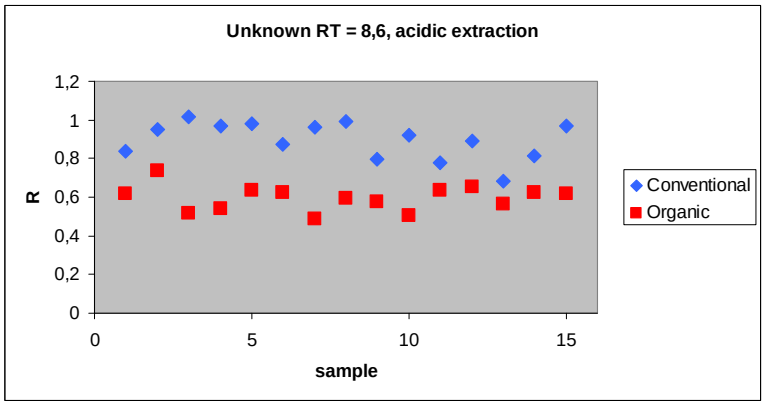


Figure 23. Relative change in content of unknown compound (RT = 8.6 min) in carrots from both agricultural systems determined after acidic extraction. Chitosan priming protocol applied in 2008 harvest season.

The data summarised in Figure 23 demonstrate discrimination between applied agricultural systems. There is a very highly significant difference between organically and conventionally grown groups of carrots ($p \leq 0.001$). Figure 24 contains the standardized amount (in mAbs*min/sample) of the unknown RT = 8.6 min for four conditions.

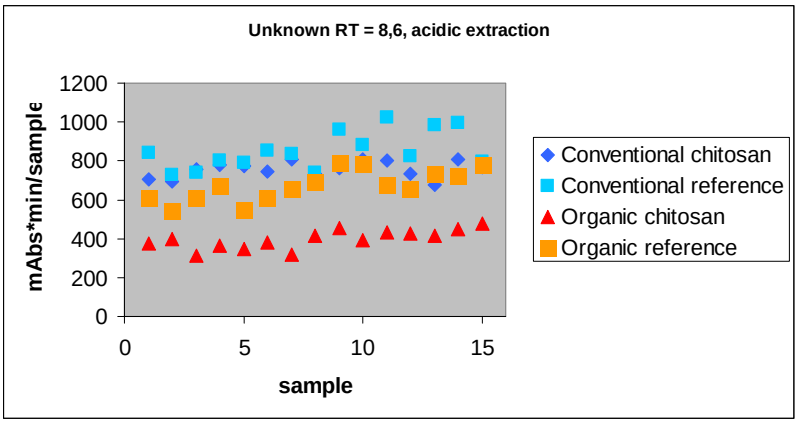


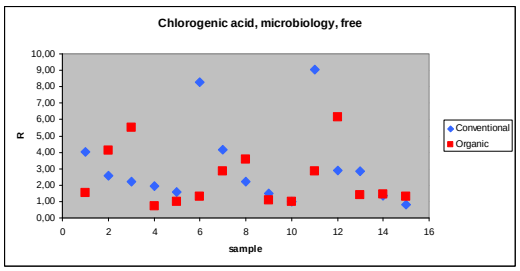
Figure 24. Standardized amount of unknown compound (RT = 8.6) estimated after acidic extraction for both agricultural system determined in the reference and chitosan primed carrot samples.

The standardized amount confirms the significant difference between both agricultural systems. The chitosan priming procedure coupled with acidic extraction and subsequent HPLC separation has the potential to discriminate between carrots grown in either an organic or a conventional agricultural system.

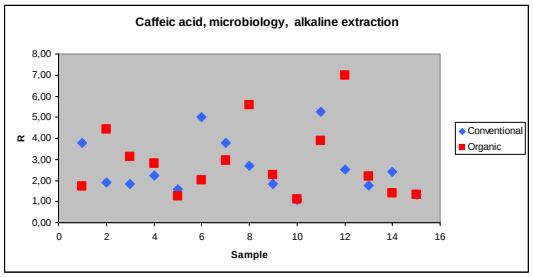
4.3.3. Microbiological inoculation

4.3.3.1. Harvest season 2007

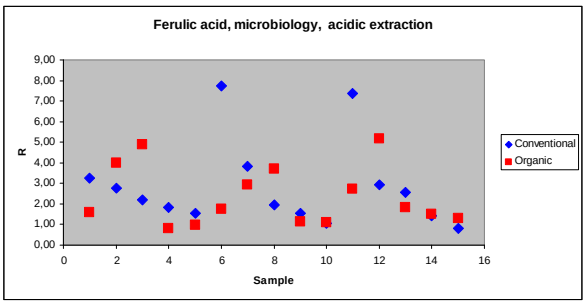
In the 2007 harvest season a 6 mm wound on carrot root was used for inoculation with selected strain of *Sclerotinia* (see Materials and methods). The impact of inoculation was evaluated in a similar way as the chemical stress.



A



B



C

Figure 25 . Relative change in phenolic acids content determined after free (A), alkaline (B) and acidic extraction (C). Carrots from 2007 harvest season inoculated with *Sclerotinia* applying a 0.6 cm wound.

In all three extraction procedures an increase in the content of phenolic acids was observed (Figure 25). Highly scattered and overlapped points did not allow discriminating between both agricultural systems. The change in phenolic acids after artificial microbiological attack is not a suitable tool for discrimination between the applied agricultural systems. Another observation concerns the change in the unknown compound (RT = 8.6 min) as is evident from Figures 26 and 27.

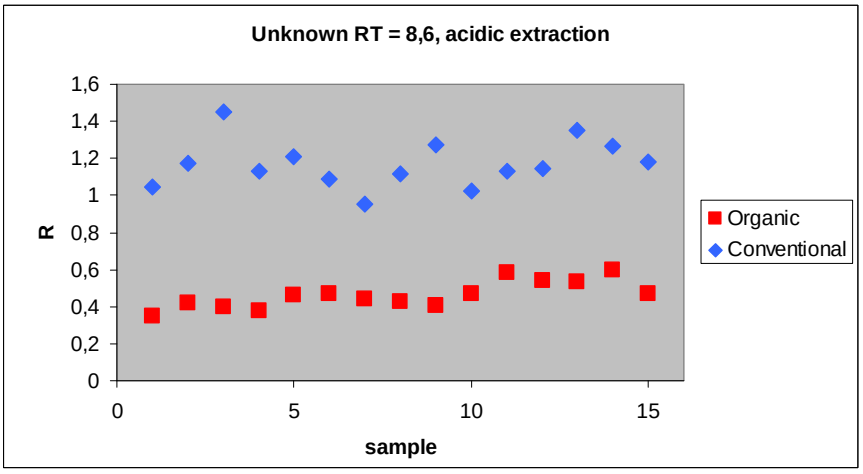


Figure 26. Relative change in content of unknown RT = 8.6 min in carrots from both agricultural systems determined after a 6 mm wound and inoculation followed with acidic extraction in 2007 harvest season.

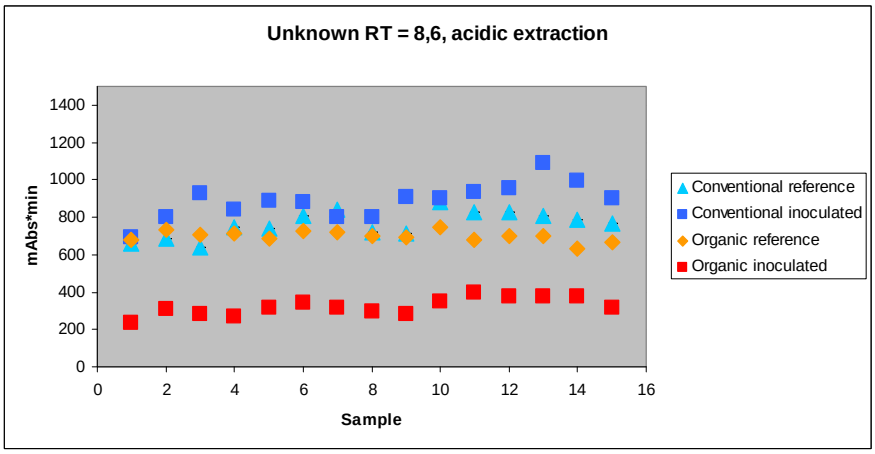
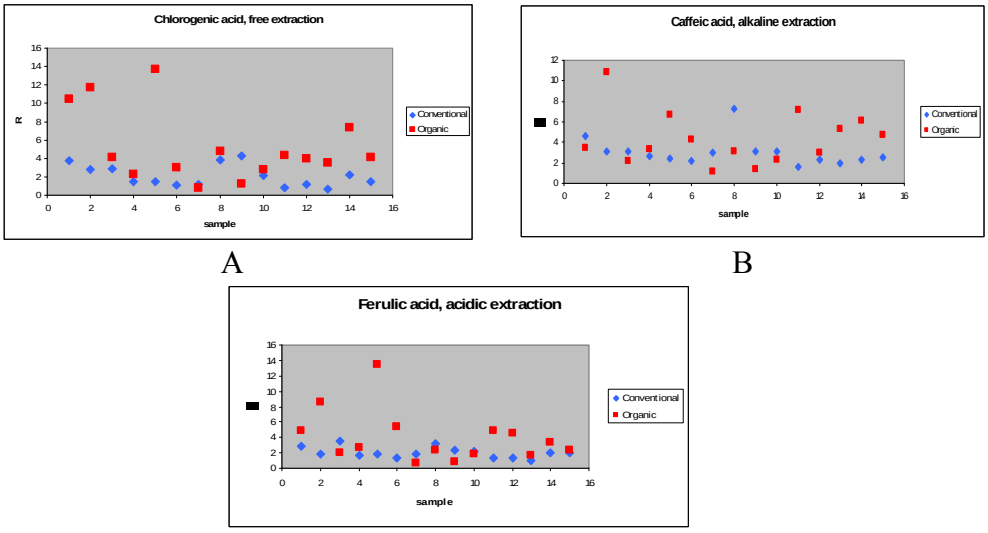


Figure 27. Standardized amount of unknown RT = 8.6 min estimated after acidic extraction for both agricultural system and reference and a 6 mm wound inoculated carrot samples from 2007 harvest season.

The data from Figure 27 demonstrate a decrease in the standardized amount of the unknown compound RT = 8.6 min in organically grown carrots. The relative number is between 0.4 and 0.6. The content of unknown RT = 8.6 min was decreased in comparison to not inoculated carrots. This is the evidence that unknown RT = 8.6 min has the origin in carrot and not in microorganisms.

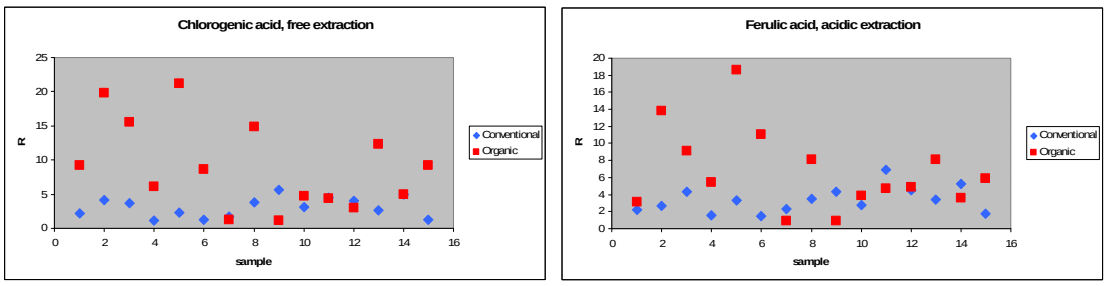
4.3.3.2. Harvest season 2008

In this harvest season two different ways of wounding (1 mm and 6 mm) coupled with inoculation with the same strain as in the 2007 harvest season were used. In case of phenolic acids contents the impact of microbiological inoculation was similar to those in the 2007 harvest season.

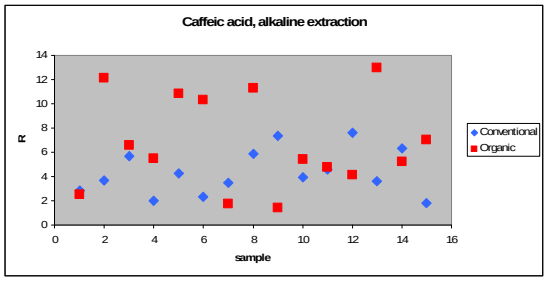


Figures 28. Relative change in phenolic acids content determined after free (A), acidic (B) and alkaline extraction (C), respectively. Carrots from 2008 harvest season with 1 mm wound and inoculation with *Sclerotinia*.

In this case an increase is also observed in all three phenolic acids – chlorogenic acid determined as free compound, ferulic acid determined after acidic extraction and caffeic acid determined after alkaline extraction. A higher variability was observed in organically grown carrots than in conventionally grown ones.



A B



C

Figures 29 . Relative change in phenolic acids content determined after free (A), acidic (B) and alkaline extraction (C). Carrots from 2008 harvest season with 6 mm wound and inoculation with *Sclerotinia*.

Similar patterns were also observed after 6 mm wound and inoculation with *Sclerotinia* in 2008 harvest season (Figure 29). The data were more scattered in organically grown carrots than in conventionally grown ones. Inoculation in 2007 harvest season provides similar scatter of phenolic acids content change between both agricultural systems (see Figures 25 A, B, C).

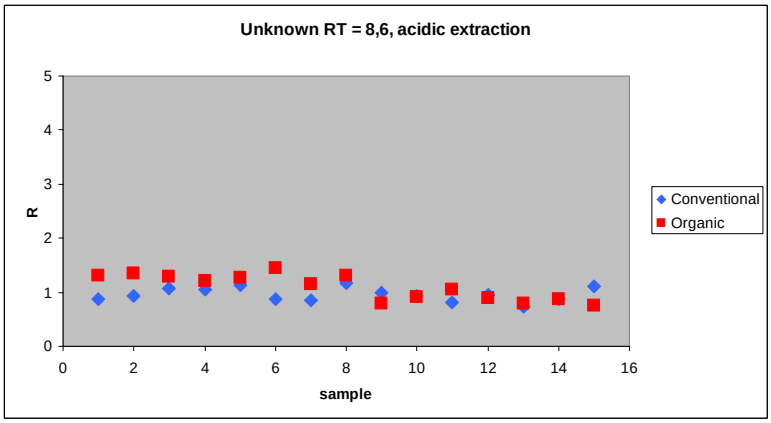


Figure 30. Relative change in content of unknown compound (RT = 8.6 min) in carrots from both agricultural systems determined after 1 mm wound and inoculation followed with acidic extraction in 2008 harvest season.

A 1 mm wound and inoculation has no effect on standardized amount of the unknown compound RT = 8.6 min. The relative change is close to 1 for both agricultural systems (Figure 30).
In case of a 6 mm wound, the changes in the amount of the unknown RT = 8.6 min are observed in Figures 31 and 32.

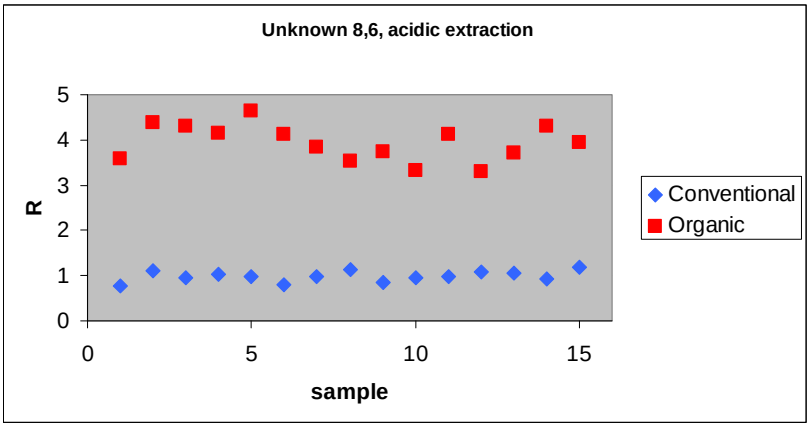


Figure 31. Relative change in content of unknown compound (RT = 8.6 min) in carrots from both agricultural systems determined after 6 mm wound and inoculation followed with acidic extraction in 2008 harvest season.

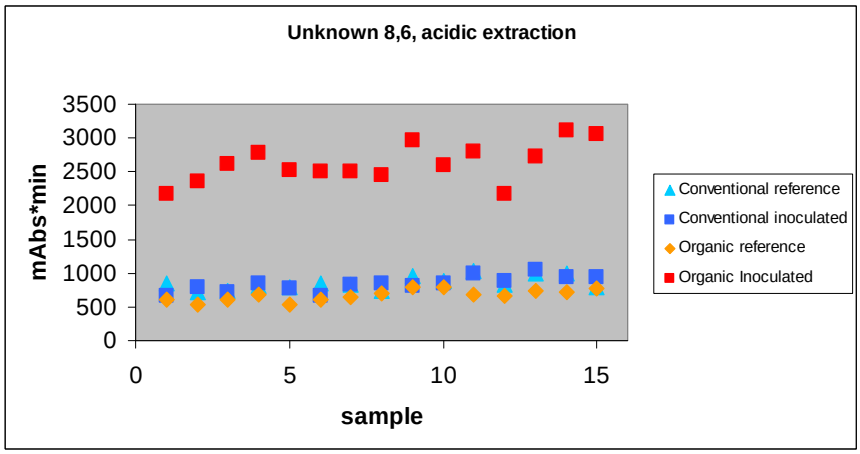


Figure 32. Standardized amount of unknown compound (RT = 8.6 min) estimated after acidic extraction for both agricultural systems in the reference and 6 mm wound inoculated carrot samples from 2008 harvest season.

From Figures 31 and 32 it is evident that the analysis by HPLC of an unknown substance with RT = 8.6 min, a 6 mm wound and inoculation of *S. sclerotiorum* offers very good discrimination between organically and conventionally grown carrots. A change was observed in the standardized amount of the unknown compound (RT=8.6 min) between the carrots in 2007 and 2008 harvest seasons. In the 2007 harvest season there is an evident drop of standardized amount in organically grown carrot while the same parameter in conventionally grown carrot demonstrates a slight increase. In the 2008 growing season there is almost no change in content of this compound in conventionally grown carrots relative to the reference one, while in organically grown carrots a remarkable increase is observed. The source of observed discrepancy could be in some changes in inoculation processes. This procedure has to be standardized.

The standardized amount of unknown compound RT = 8.6 min was in the 2008 harvest season shifted to the levels above 2000 units in organically grown carrot. In 2007 harvest season the standardized amount of this compound dropped to levels below 400 units. Due to this remarkable shift we wanted to see if during the analysis within the years 2005

– 2008 a standardized amount of unknown RT = 8.6 at levels more than 2000 units or lower than 400 units was observed.
We have checked all data from 2005 – 2008 harvest seasons and also data for unknown carrots taken from the local marked without knowing their variety and history of treatment received after acidic extraction. We have focused our attention on the unknown compound (RT = 8.6 min). In all cases the standardized amount of this compound was within the range of 550 and 1200 standardized amount units. The presence of unknown compound (RT = 8.6 min) is always within this range also for BABA or chitosan primed as well as inoculated conventional carrots as we have demonstrated with our results presented here. Only after hard inoculation (6 mm wound) of organically grown carrot the presence of this compound shifts out of the range 550 – 1200 standardized amount units. The unknown compound RT = 8.6 offers a clear separation between conventionally and organically grown carrots after microbiological perturbation.
Inoculation followed with acidic extraction and estimation of standardized amount of the unknown compound RT = 8.6 min has good potential to be a suitable tool for discrimination organically and conventionally grown carrots. The whole procedure has to be standardized and the unknown compound RT = 8.6 min identified.

5. CONCLUSION

In this exploratory research project we focused on plants' defence system as a possible source of discrimination between organically and conventionally grown carrots. We performed a metabolome fingerprint comparison of ^{1}H NMR spectroscopy data and ultraperformance liquid chromatography coupled to mass spectroscopy. JRC – IHCP is currently finalising all the statistical analyses. Nonetheless, the metabolome fingerprint related to UPLC MS application seems to be promising based on the first data (Figure 16). ^{1}H NMR does not offer so straightforward optimism. At this stage, it cannot be said if the compounds with a potential for discrimination found by UPLC MS are related to plant defence system. These compounds have to be identified and experiments verified on more samples of the selected biological model – i.e. carrot.

Microbiological attack and subsequent estimation of the lesion size in plants due to *Sclerotinia sclerotiorum* from both agricultural systems does not give potential for discrimination. Primed carrots did not shown differences in lesion size between both agricultural systems. However, the problem related to certain overlap between both groups remains.

Metabonomics seems to have potential for discrimination. Priming with BABA or chitosan has a positive effect on the amount of phenolic acids content, the most important metabolites in plant defence response, but no positive impact on discrimination of both agricultural systems was observed. It seems that selected phenolic acids are not suitable markers for the identification of the origin of production systems, per se. However, when acidic extraction was applied there was a, so far unidentified, unknown compound with a retention time RT = 8.6 min, which offers potential for discrimination of organically grown carrots from conventionally grown ones. It was confirmed that this compound has an origin in carrots. It was identified also in acidic extracts of reference carrots. In case of priming with BABA the t-test showed a p value = 0.116 while in priming with chitosan p value of the t-test was $8.97.10^{-11}$. There is an interesting potential to use chitosan priming procedure for discrimination between organically and conventionally grown carrots. However, it is necessary to verify its effect on various carrots varieties grown under several environments focusing on increasing the difference between both agricultural systems.

In our study, microbiological inoculation with *Sclerotinia sclerotiorum* followed by determination of the unknown compound (RT=8.6 min) by HPLC offers the best discrimination between agricultural systems, although discrepancies were observed in the carrots' reaction in 2007 and 2008 harvest season. This has to be solved focusing on standardization of the inoculation process. In both cases 6 mm wounding and inoculation offers very good separation between organically and conventionally grown carrots. No overlap was observed for each year. After checking the data from various years of analysis and using different varieties of carrots from the market it is clear, that the standardized amount of unknown RT = 8.6 ranges between 550 and 1200. After inoculation with microorganisms the standardized amount of this compound shifts to safe distance from this range in organically grown carrots.

We have proven that a difference between organically and conventionally grown carrots which was not clearly observed up to now based on published literature data, can be observed. It would be then possible to discriminate both agricultural systems just based

on this compound. This gives a great potential for application of this method in routine work.
To really apply this method in a medium-term it is necessary to identify the unknown compound with a retention time (RT) of 8.6 min. Knowing this molecule it will be possible to carry out experiments focusing on the physiological role of this compound in carrot. It is also important to identify this compound for quantification purpose. In addition to this requirement is also critical to standardize the microbiological inoculation process.
Finally the unknown RT = 8.6 min was also identified in winter wheat so the potential for other crops is opened.

6. LITERATURE

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7. ANNEX

ANNEX 1. Identification and handling of the carrot samples.

Sample_ID IHCP	Sample_ID IRMM	Agric. Syst.	Env.	Sampling date	Storage time at manufacture (month)	Lyophilisation date	Storage time in the freezer at -20 °C (month)	Sample Labelling
09010007	5202 - 5206	O	2	03/10/07	0	10/10/07	16	Fresh
09010008	5238 - 5242	O	2	03/10/07	0	10/10/07	16	Fresh
09010009	5274 - 5278	O	2	03/10/07	0	10/10/07	16	Fresh
09010010	5311 - 5315	C	2	03/10/07	0	10/10/07	16	Fresh
09010011	5347 - 5351	C	2	03/10/07	0	10/10/07	16	Fresh
09010012	5383 - 5387	C	2	03/10/07	0	10/10/07	16	Fresh
09010013	4979 - 4983	O	1	01/10/07	0	10/10/07	16	Fresh
09010014	5016 - 5020	O	1	01/10/07	0	10/10/07	16	Fresh
09010015	5053 - 5057	O	1	01/10/07	0	10/10/07	16	Fresh
09010016	5129 - 5133	C	1	01/10/07	0	10/10/07	16	Fresh
09010017	5166 - 5170	C	1	01/10/07	0	10/10/07	16	Fresh
09010018	5092 - 5096	C	1	01/10/07	0	10/10/07	16	Fresh
09010114	5633	C	1	29/09/08	4	23/01/09	0	Stored
09010115	5634	C	1	29/09/08	4	23/01/09	0	Stored
09010116	5635	C	1	29/09/08	4	23/01/09	0	Stored
09010117	5667	O	1	29/09/08	4	23/01/09	0	Stored
09010118	5668	O	1	29/09/08	4	23/01/09	0	Stored
09010119	5669	O	1	29/09/08	4	23/01/09	0	Stored
09010120	5686	C	2	29/09/08	4	23/01/09	0	Stored
09010121	5687	C	2	29/09/08	4	23/01/09	0	Stored
09010122	5688	C	2	29/09/08	4	23/01/09	0	Stored
09010123	5705	O	2	29/09/08	4	23/01/09	0	Stored
09010124	5706	O	2	29/09/08	4	23/01/09	0	Stored
09010125	5707	O	2	29/09/08	4	23/01/09	0	Stored
09010126	5709	C	1	29/09/08	0	06/10/08	5	Fresh
09010127	5710	C	1	29/09/08	0	06/10/08	5	Fresh
09010128	5711	C	1	29/09/08	0	06/10/08	5	Fresh
09010129	5712	O	1	29/09/08	0	06/10/08	5	Fresh
09010130	5713	O	1	29/09/08	0	06/10/08	5	Fresh
09010131	5714	O	1	29/09/08	0	06/10/08	5	Fresh
09010132	5715	C	2	29/09/08	0	06/10/08	5	Fresh
09010133	5716	C	2	29/09/08	0	06/10/08	5	Fresh
09010134	5717	C	2	29/09/08	0	06/10/08	5	Fresh
09010135	5718	O	2	29/09/08	0	06/10/08	5	Fresh
09010136	5719	O	2	29/09/08	0	06/10/08	5	Fresh
09010137	5720	O	2	29/09/08	0	06/10/08	5	Fresh

ANNEXES

ANNEX 1: Guidelines and forms: applications for new projects

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Scientific committee

Call for proposals

Exploratory Research 2008

Guidelines

Layout

- A proposal should be submitted before 17:00 September 14th, 2007 using the attached application form "Call for proposals - Exploratory research 2008". Proposals submitted after this deadline will not be considered.
- A proposal should contain a maximum of five pages (approval page not included).
- A proposal should be submitted both in an electronic form to the secretariat of the Scientific Committee (ursula.vincent@ec.europa.eu) and as a signed paper copy by internal post to Ursula Vincent.

A proposal should contain the following information:

1. Project co-ordinator
2. Title of project
3. State-of-the-art
4. Rationale[1]
5. Objectives[2]
6. Scientific novelty of the project
7. Why should the research be carried out as exploratory research and not in the framework institutional work?

[1] The rationale should be introduced e.g. as follows: "there is a need for...", "there is a problem with...", "European legislation requires that...", etc.

[2] The objectives should therefore be presented e.g. as follows: "as a consequence of the rationale, the objectives are: to develop..., to produce..., to provide...," etc.

8. Why should the JRC and in particular the IRMM carry out the project?
9. Work to be carried out, research methods/techniques, and list of expected deliverables (publications, procedures, software, patents, etc). The proposal is expected to contain a work plan and schedule, an identification of critical points in the project and suggestions of alternatives in case of failure, if possible.
10. Brief description of the scientific background of the team members (including e.g. reference to relevant publications).
11. Name of institutional action to which the research proposal is linked and a brief description of the nature of the link
12. Possible follow-up (e.g. institutional, competitive projects)
13. Resources: specific credits and person months. Specific credits should be fully justified.
14. Signature of unit head(s) (only for paper copy).

Guidance

- There should be a clear element of scientific research in the proposal; state-of-the-art studies are not eligible.
- An ER proposal should **not** immediately be interchangeable with an institutional project.
- It should allow the scientific risk inherent to all fundamental research.
- The proposal should clearly display the novelty components.
- The call is open to all IRMM scientific members of staff.
- Feasibility is a requirement (appropriate allocation of time, budget, staff)
- The criteria for selection are as follows (weighting factors are given in brackets):
    1. Relevance to Institute's scientific activities and the JRC mission **(0.1)**
    2. Scientific novelty (including new approaches) of the proposed research **(0.4)**
    3. Quality of research plan (including in particular the adequacy of methods/techniques, the resources (human and budgetary) and the time schedule). Proposals involving the use of equipment of different units should clearly state a user planning **(0.3)**
    4. Contribution to integrate resources (e.g. inter-units projects, inter-institutes projects) and networking **(0.2)**

- The selection procedure will be performed as follows:

  1) Evaluation of the written proposals by the IRMM Scientific Committee,

  2) Auditions of the candidates by the IRMM Scientific Committee and

3) Final evaluation and selection of the proposals.

- For ER 2008 projects, the schedule will be as follows:
    - Launch of call: 1st June 2007
    - Deadline for submission of written proposals: 14th September 2007
    - Auditions of all applicants: 1st October 2007
    - Final selection meeting of the SC: 2nd October 2007
    - Deadline for the communication of the list of selected proposals: 8th October 2007
- The work should be carried out at IRMM, with existing major equipment.
- Each project should clearly identify one project co-ordinator. The project co-ordinator is responsible for the smooth running of the project. He/she should therefore be working at IRMM during the duration of the whole project.
- Joint proposals between IRMM units and/or external partners (e.g. other JRC institutes) are strongly encouraged. To qualify as a joint proposal the contribution of each unit/partner has to represent a minimum of 10% of resources.
- The proposal should only cover work to be carried out from January to December 2008.
- The selected applicants will be invited to present their results during an IRMM workshop organised during the first quarter of 2009.
- The selected applicants should also submit a final report of their research in the course of the second quarter of 2009.

Resources

The JRC can spend up to 6% of its budget on Exploratory research. For IRMM, this means a total of 10-12 person years and about 300 000 euros in specific credits. A typical project could therefore include about 1-3 person year and specific credits of up to 50 000 euros. These additional credits will be specifically reserved for each action involved.

Important Note

Please be reminded that a Hazard Identification and Risk Assessment is (legally) required for all new (and amended) projects starting in IRMM. Refer to IRMM PR-0022 and F-0033.

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Scientific committee

Call for proposals

Exploratory Research 2008

Application form

Project number: ER/2008/

To be sent to the secretariat of the SC **before September 14th 2007**

1. Project co-ordination and application details

Name of project co-ordinator:
IRMM Unit(s) involved:
External partners (if applicable):

2. Title of the project

3. State-of-the-art

4. Rationale

5. Objectives

6. Scientific novelty of the project

7. Why should the research be carried out as exploratory research and not in the framework institutional work?

8. Why should the JRC and in particular the IRMM carry out the project?

9. Work to be carried out, research methods/techniques, and list of expected deliverables (publications, procedures, software, patents, etc). Work plan and schedule, identification of critical points and suggestions of alternatives in case of failure, if possible.

10. Brief description of the scientific background of the team members (including e.g. references to relevant publications)

11. Name of the institutional action(s) to which the research proposal is linked and a brief description of the nature of the link

12. Possible follow-up (e.g. competitive projects)

13. Resources: specific credits and staff allocation

13.a. specific credits

Specify costs and allocate credits to the relevant action(s).

13.b. staff * allocation in person months (* project co-ordinator to be working at IRMM during 2008)

14. Approval of unit head(s)

Name:	Date:	Signature:

ANNEX 2: GUIDELINES AND FORMS: APPLICATIONS FOR PROLONGATION OF PREVIOUS PROJECTS

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Scientific committee

Call for prolongation of previous projects

Exploratory Research 2008

Guidelines

Layout

- A proposal should be submitted before 17:00 September 14th, 2007 using the attached application form "Call for prolongation of previous projects - Exploratory research 2008". Proposals submitted after this deadline will not be considered.
- A proposal should contain a maximum of five pages (approval page not included).
- A proposal should be submitted both in an electronic form to the secretariat of the Scientific Committee (ursula.vincent@ec.europa.eu) and in a signed paper copy by internal post to Ursula Vincent.

A proposal should contain the following information:

1. Project co-ordinator
2. Title of project (should be different from that of the initial project)
3. Summary of the outcome and deliverables of the initial project
4. Objectives of the prolongation (including scientific novelty)
5. Why should the research **still** be carried out as exploratory research and not in other frameworks (e.g. institutional work, competitive actions)?
6. Work to be carried out, research methods/techniques, and list of expected deliverables (publications, procedures, software, patents, etc). The proposal is expected to contain a work plan and schedule, an identification of critical points in the project and suggestions of alternatives in case of failure, if possible**.**
7. Name of institutional action to which the research proposal is linked

8. Possible follow-up (e.g. institutional, competitive projects)
9. Resources: specific credits and person months. Specific credits should be fully justified.
10. Signature of the unit head(s).

Guidance

- There should be a clear scientific justification in the application for the prolongation.
- "Application for the prolongation of a previous exploratory research project" **does not mean** "extension of time to finish a previous project" with the same working plan. It means looking for further exploratory developments, with further objectives and a new research plan.
- The call is open to IRMM scientific staff members who were laureates of the previous ER call.
- Feasibility is a requirement (appropriate allocation of time, budget, staff).
- The criteria for selection are as follows (weighting factors are given in brackets):
    1. Justification for the prolongation in the frame of ER **(0.25)**
    2. New objectives and scientific novelty **(0.3)**
    3. Quality of research plan (including in particular the adequacy of the methods/techniques, the resources (human and budgetary) and the time schedule). Proposals involving the use of equipment of different units should clearly state a user planning **(0.3)**
    4. Contribution to integrate resources (e. g. inter-units projects, inter-institute projects) and networking **(0.15)**

- The selection procedure will be performed as follows:

  1) Evaluation of the written proposals by the IRMM Scientific Committee,

  2) Auditions of the candidates by the IRMM Scientific Committee and

  3) Final evaluation and selection of the proposals.

- For ER 2008 projects, the schedule will be as follows:
    - Launch of call: 1st June 2007
    - Deadline for submission of written proposals: 14th September 2007
    - Auditions of all applicants: 1st October 2007
    - Final selection meeting of the SC: 2nd October 2007
    - Deadline for the communication of the list of selected proposals: 8th October 2007

- The work should be carried out at IRMM, with existing major equipment.
- Each project should clearly identify one project co-ordinator. The project co-ordinator is responsible for the smooth running of the project. He/she should therefore be working at IRMM during the duration of the whole project.

- Joint proposals between IRMM units are strongly encouraged. To qualify as a joint proposal the contribution of each unit/partner has to represent a minimum of 10% of resources.
- The proposal should only cover work to be carried out from January to December 2008.
- The selected applicants will be invited to present their results during an IRMM workshop organised during the first quarter of 2009.
- The selected applicants should also submit a final report of their research in the course of the second quarter of 2009.

Resources

The JRC can spend up to 6% of its budget in Exploratory research. For IRMM, this means a total of 10-12 person years and about 300 000 euros in specific credits. A typical project could therefore include 1-3 person year and specific credits up to 50 000 euros. These additional credits will be specifically reserved for each action involved.

Important Note:

Please be reminded that a Hazard Identification and Risk Assessment is (legally) required for all new (and amended) projects starting in IRMM. Refer to IRMM PR-0022 and F-0033. A new Hazard Identification and Risk Assessment does not need to be carried out for a prolongation without any project amendments.

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Scientific committee

Call for prolongation of previous projects

Exploratory Research 2008

Application form

Project number: ER/2008/

To be sent to the secretariat of the SC **before September 14th 2007**

1. Project co-ordination and application details

Name of project co-ordinator:
IRMM Unit(s) involved:
External partners (if applicable):

2. Title of project (should be different from that of the initial project)

3. Summary of the outcome and deliverables of the initial project

4. Objectives of the prolongation (including scientific novelty)

5. Why should the research still be carried out as exploratory research and not in other frameworks (institutional work, competitive actions)?

6. Work to be carried out, research methods/techniques, and list of expected deliverables (publications, procedures, software, patents, etc). Work plan and schedule, identification of critical points and suggestions of alternatives in case of failure, if possible.

7. Name of the institutional action(s) to which the research proposal is linked

8. Possible follow-up (e.g. competitive projects)

9. Resources: specific credits and staff allocation

9.a. specific credits

Specify costs and allocate credits to the relevant action(s).

9.b. staff * allocation in person months (*project co-ordinator to be working at IRMM during 2008)

10. Approval of unit head(s)

Name:	Date:	Signature:

ANNEX 3: SELECTED PROJECTS FOR THE YEAR 2008

Project		Action
Title	**Co-ordinator**	
Neutron elastic scattering measurements in time of flight at GELINA	A Plompen	51402
Coupling of High Performance Liquid Chromatography to Mass Selective Detection by Atmospheric Pressure Laser Ionisation to Determine 15+1 EU Priority PAHs in Food	R. Simon	33004
Towards the metrological basis for measurements of size and shape of proteins under physiological conditions – Not done	G. Roebben	11102
Method development for quantitative PFOS analysis: preparing for the future – Not done	S. Voorspoels	22006
Development and optimization of a method for quantification of fishmeals in feeds by real-time PCR	M. Prado Rodríguez	33002
Dual isotope analysis of nitrate in seawater, fresh water and wastewater using chemical reduction to N2O	F. Accoe	22007
Feasibility study: Assessment of the influence of the uncertainty components involved in the determination of low DNA copy numbers – Not done	D. Gancberg	15012
Detection of potentially allergenic peptides derived from bovine milk enzymatic hydrolysates in food matrices by a proteomic approach	V. Trégoat	33004
Exploratory study on the application of metabolomic and metabonomic for the discrimination of organically and conventionally grown carrots	A. Maquet	33004
Production of double deposition uranium particle reference materials, including method development for TIMS measurements of single particles	Y. Aregbe	53102

European Commission

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