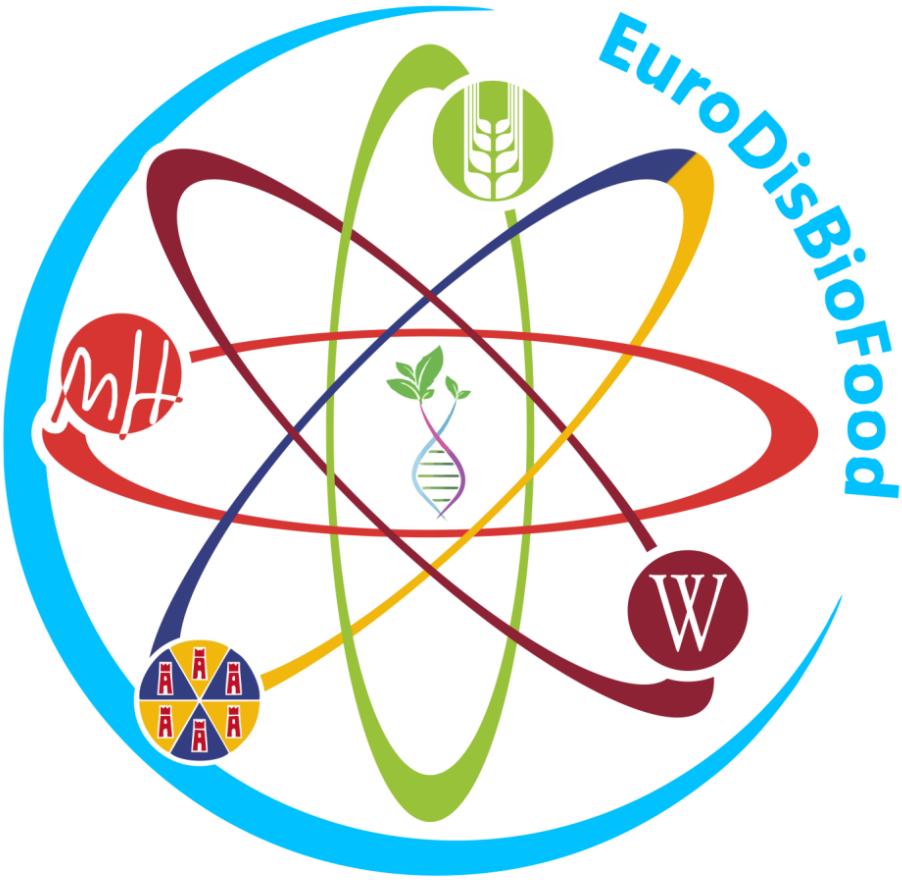

Modern Methodological Procedures in Science I. – IV.

A Methodological Handbook for PhD students

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Modern Methodological Procedures in Science I. and IV.

PARTS: I. BIOLOGY, II. BIOTECHNOLOGY, III. FOOD SCIENCES and IV. FOOD TECHNOLOGY

A Methodological Handbook for PhD students

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CONTENT

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PARTS: I. BIOLOGY and II. BIOTECHNOLOGY

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1. ABTS total antioxidant capacity assay

EQUIPMENT AND REAGENTS:

Equipment and reagents	Description
10 ml beaker	Sigma-Aldrich
Kern ABT analytical balances	Sigma-Aldrich
Redistilled water	
ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)	Sigma-Aldrich
Potassium persulphate ($K_2S_2O_8$)	Sigma-Aldrich
Sodium hydroxide (NaOH)	Sigma-Aldrich
Acetone	Sigma-Aldrich
Ethanol (96-100 %)	Sigma-Aldrich
Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)	Sigma-Aldrich
Pipettes and pipette tips	Sigma-Aldrich
Laboratory Centrifuge	Thermo Scientific™
Semi-micro cuvettes	Fisher Scientific
6705 UV/Vis Spectrophotometer, Jenway	Fisher Scientific

METHOD DESCRIPTION:

In methods, which have been developed to measure Total Antioxidant Capacity – TAC, generally, a type of radical is generated in the assay and the antioxidant activity of the sample against the radical is measured. The most widely used colorimetric methods is 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) $ABTS^{\cdot+}$ - based method, in which a colorless molecule, reduced ABTS, is oxidized to a characteristic blue-green $ABTS^{\cdot+}$. When the colored $ABTS^{\cdot+}$ is mixed with any substance that can be oxidized, it is reduced to its original colorless ABTS form again; in contrast, the reacted substance is oxidized. This feature is the basic principle of the methods that use ABTS. Various oxidants have been used to oxidize ABTS molecules such as potassium persulfate.

The ABTS assay was first reported by Miller et al. (1993) and has been improved and widely used in testing antioxidant capacity in food samples.

The ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)) is a peroxidase substrate, which when oxidized by peroxyl radicals or other oxidants in the presence of H_2O_2 generates a metastable radical cation ($ABTS^{\cdot+}$) which is intensely colored and can be monitored spectrophotometrically in the range of 600 – 750 nm. The antioxidant capacity is measured as the ability of test compounds to decrease the color reacting directly with $ABTS^{\cdot+}$ radical and expressed relative to Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analog of vitamin E. (Roginsky and Lissi, 2005).

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The advantages of using this method for antioxidant capacity assay is that $ABTS^{\cdot+}$ can be solubilized in both aqueous and in organic media and is not affected by ionic strength, so the antioxidant capacity can be measured due to the hydrophilic and lipophilic nature of compounds (Arnao, 2000).

ASSAY PROTOCOL:

1. Use two types of sample extraction - hydrophilic and lipophilic to assess the antioxidant power of fruit/vegetable samples.
2. For hydrophilic extraction:
    - Weigh 1 g of homogenized fruit/vegetable sample and dilute it with ethanol and water (80% v/v) to a final ratio of 1:10 for weight to volume.
    - Adjuste the pH of each sample with 1 mol/L of NaOH until it reached 5.0 ± 0.2.
    - Centrifuge for 10 min at 2000*g*.
    - Supernatant of each sample use for measurement (Scalzo et al., 2005).
3. For lipophilic extraction:
    - Add acetone (ratio of 1:2 or 1:5 for weight to volume according to type of fruit).
    - Centrifuge for 15 min at 2000*g*.
    - Supernatant of each sample use for measurement (Scalzo et al., 2005).
4. Prepare solutions: 7 mmol/L ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) and 2.45 mol/L potassium persulphate ($K_2S_2O_8$). ABTS reacts with potassium persulfate in a stoichiometric ratio of 2: 1, excess ABTS is important to maintain the stability of the reaction mixture. The radical cation ($ABTS^{\cdot+}$) is generated for 12-16 hours in a dark place at laboratory temperature.
5. Prepare the ABTS·+ working solution - dilute the solution of $ABTS^{\cdot+}$ using 95 % ethanol to concentration of solution with absorbation 0,700 ± 0,02 at wavelength 734 nm.
6. Add 10 µL of sample to 990 µL of $ABTS^{\cdot+}$ working solution in semi-micro cuvette and mix well. Use 95 % ethanol as a reference solution for measurement.
7. Record absorbance at 734 nm measured in a spectrophotometer in time 0 and 10 minutes. The result of absorbation is calculated: $A = A_{10min} - A_{0min}$.
8. Expresse the antioxidant abilities of sample as Trolox Equivalent Antioxidant Capacity (TEAC) by using the calibration curve plotted against different amounts of Trolox. TEAC values are calculated and expressed using Trolox equivalents (TE) per gram of fresh weight (FW). Data are expressed as antioxidant capacity induced by hydrophilic and lipophilic components, and total antioxidant capacity as the sum of the two phases.

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2. Acid polyacrylamide gel electrophoresis

EQUIPMENT AND REAGENTS:

Equipment	
Equipment	Description
Vertical electrophoretic unit	Hoefer SE 600 Series
Scaner Bio-Rad GS-800 Calibrated Densitometer	
Pipettes	Range from 1 µl to 10000 µl
Reagent and Materials	
2-chloroethanol	SIGMA
pyronin G	SIGMA
Glacial acetic acid	SIGMA
Glycine	SIGMA
Acrylamide	SIGMA
Urea	SIGMA
Bisacrylamide	SIGMA
Ascorbic acid	SIGMA
Ammoium persulphate	SIGMA
TEMED	SIGMA
$FeSO_4$	SIGMA
Spectra Multicolor Broad Range Protein Ladder	Thermo Scientific
Gloves	
Sterile filter tips	10mL, 1000µL, 200 µL, 10 µL
Microtubes	1.5ml microtubes

METHOD DESCRIPTION:

Acid electrophoresis distributes proteins based on size and charge molecules. This method is mainly used for the analysis of gliadin fractions and also glutenin proteins in individual cereal varieties.

ASSAY PROTOCOL:

1. **Prolamin extraction**

Extraction procedure:

a) 1 mg of homogenized grain add to 0,005ml extraction solution for 24h laboratory temperature.

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b) Centrifuge for 10 min at 15 000 rpm at 4 °C.
c) Supernatant transfer to new tube.

2. Prolamin acid electrophoresis

a) Preparing of the solutions:

Gel buffer solution:

Glacial acetic acid	2,0 ml
Glycine	0,10 g
H_2O	up to100 ml

Storage solution:

Acrylamide	10 g
Urea	6 g
Bisacrylamide	0,4 g
Ascorbic acid	0,1 g
Gel buffer solution	up to 100 ml

Electrophoretic buffer:

Glacial acetit acid	8 ml
Glycine	8 g
H_2O	up to 2 L

b) Preparation of the 1 mm polyacrylamide gel:

Storage solution	99,10 ml
5% Ammoium persulphate	600 µl
TEMED	300 µl
$FeSO_4$	0,3 ml (5000 mg $FeSO_4$ + 40 ml H_2O + 6 drops H_2SO_4)

c) *Electrophoretic separation procedure*

30 min	20 mA	130 V	50 W
45 min	30 mA	380 V	50 W
8 hours	30 mA	500 V	50 W

d) *Gel staining*

Solutions:

0,5 % Coomasie briliant blue R250	0,5 g CBB R250 up to 100 ml ethanol
10 % trichloroacetic acid	200 g trichloroacetic acid up to 100 ml H_2O

Staining solution:

190 ml	10 % trichloroacetic acid
10 ml	0,5 % Coomasie briliant blue R250

Staining procedure:

Gel immerse for 24 h in laboratory temperature in staining solution

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e) *Gel destaining*

Destaining solution:

Acetic acid	200 ml
Glycerol	40 ml
H_2O	up to 2 L

Destaining procedure:

Gel immerse for 24 h in laboratory temperature in destaining solutio

INTERPRETATION OF THE RESULTS:

Interpretation of results is based on comparison of the separated molecules in gel according to standard sample and according to marker of molecular weight by software. Using electrophoretic separation of proteins in an acidic conditions (A-PAGE) is obtained the electrophoretic spectrum of proteins of individual crop species. By evaluating the electrophoreograms, the percentage of prolamine fractions, which were separated in the gel into α-, β-, γ- and ω-prolamins, can be calculated. Based on the electrophoretic mobility of the proteins in an acidic conditions were at the top of the gel separated ω-prolamins, followed by γ-prolamins, in the middle part β-prolamins and in the lower parts of the α-prolamin in the polyacrylamide gel. Separated protein molecules is possible to compare within two or more samples which obtain differentiation of samples. There is also possibility to investigated changes in proteome of individual sample in different conditions.

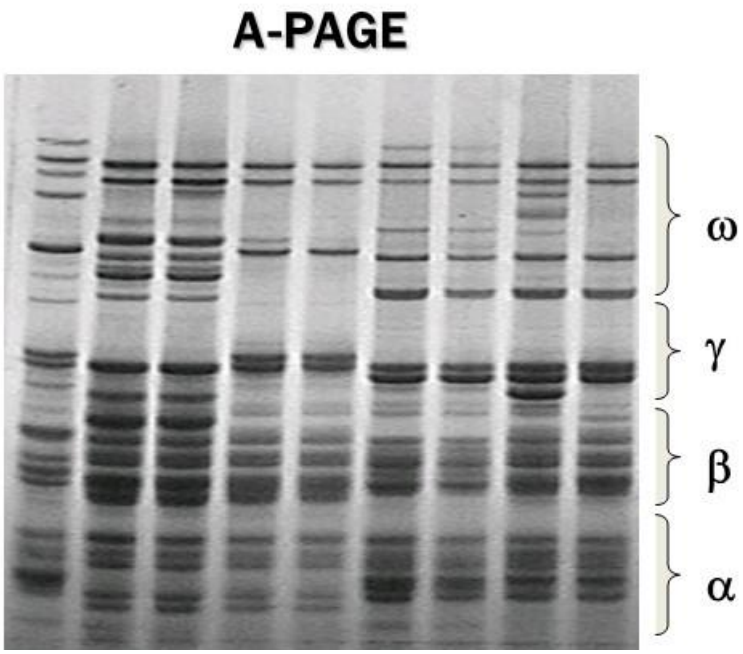

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Fig. A-PAGE electrophoreogram

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3. Acrosomal status of mammalian spermatozoa: a single stain method

EQUIPMENT AND REAGENTS:

Equipment	
Equipment	Description
Glass slides	75 x 26 x 1 mm
Micro pipettes	Range from 2 µL to 20 µL
Reagent and Materials	
Rose Bengal	(Dye content 95%)
Fast Green FCF	(Dye content ≥85%)
Ethanol	40%
Citric acid	
Disodium phosphate buffer	
1.5 mL tubes	

METHOD DESCRIPTION:

Staining solution preparation

The staining solution is comprised of 1% (w/v) rose Bengal solution, 1% (w/v) fast green FCF and 40% ethanol in citric acid (0.1 M)-disodium phosphate (0.2 M) buffer.

Acrosome staining

Add 20 µL of staining solution to 20 µL of diluted semen (10×10^6 spermatozoa/mL) in a 1.5 mL tube. After 70 seconds at room temperature, put 10 µL of the mixture onto the glass slide and smear the mixture evenly over the entire surface of slide using another glass slide. Let the glass slide dry on air at room temperature.

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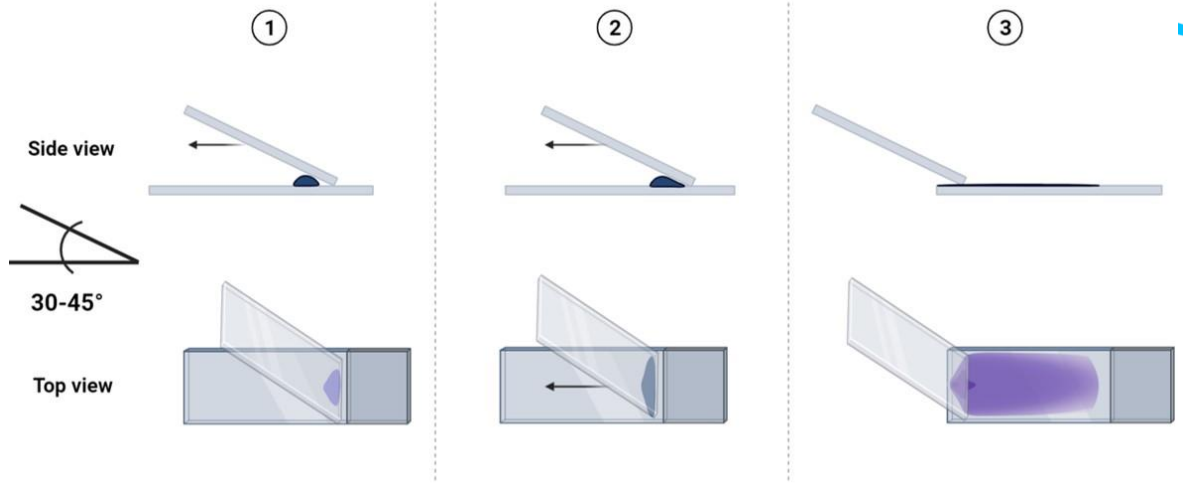
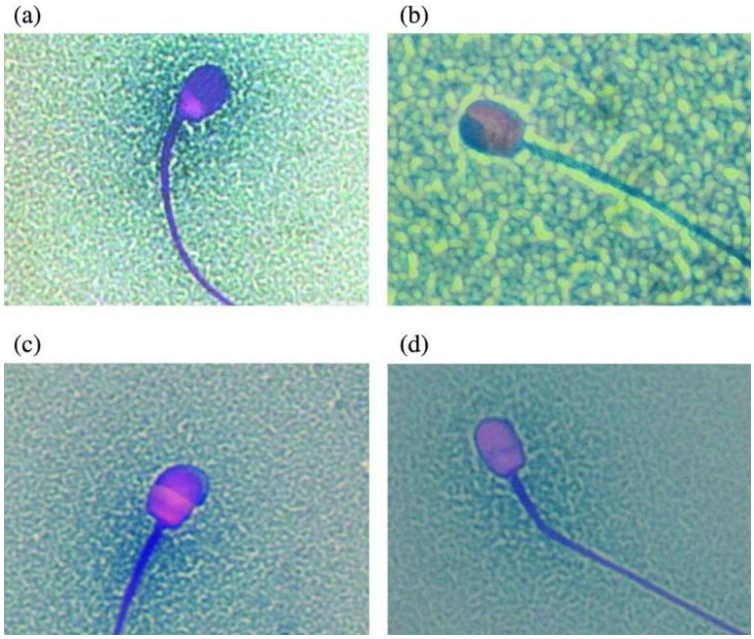


Acrosome status evaluation:

At least 200 cells should be examined under bright-field microscope at magnification of × 630. There are several quality categories including:

a) intact acrosome
b) damaged acrosome membrane
c) separated from the sperm head
d) missing acrosome



Express the result as the percentage of spermatozoa with damaged/undamaged acrosome.

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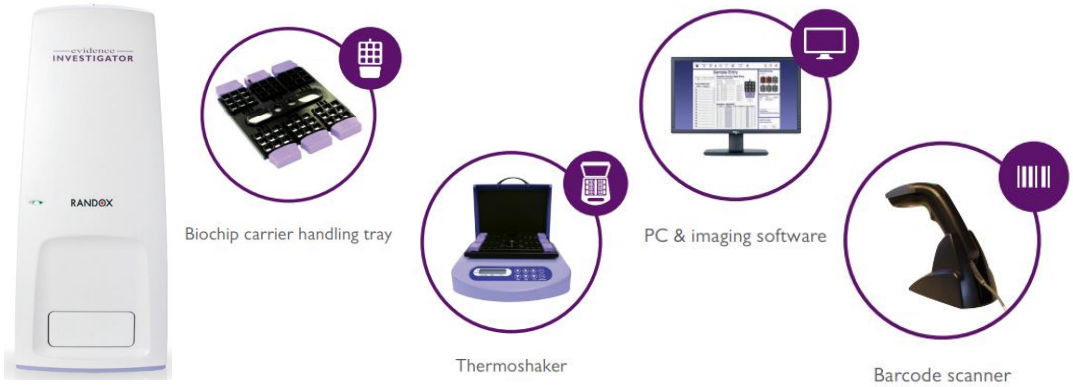
4. Biochip Array Technology - multiplexed measurement of cytokines in blood serum with a high sensitivity evidence biochip array

EQUIPMENT AND REAGENTS:

Equipment and reagents - not supplied with the kit	
Equipment	Description
The Evidence Investigator (Randox Laboratories Ltd.; Crumlin, United Kingdom) comes supplied as part of a package, with all essential components provided. These components are approved for use with the Evidence Investigator and make it easier for the user to conduct testing. 	
Micro pipettes	Range from 100 µL to 1000 µL
Reagent and Materials	
Cytokine Array I kit	Cat. No. EV 3623; Randox Laboratories Ltd.; Crumlin, United Kingdom
High Sensitivity Cytokine Array I Control	Cat. No. CY5005; Randox Laboratories Ltd.; Crumlin, United Kingdom

METHOD DESCRIPTION:

The Evidence Investigator™ Biochip Array technology is used to perform simultaneous quantitative detection of multiple analytes from a single sample. The core technology is the Randox Biochip, a solid-state device containing an array of discrete test regions of immobilised antibodies specific to different cytokines and growth factors. A sandwich chemiluminescent immunoassay is employed for the cytokine array. Increased levels of cytokine in a specimen will lead to increased binding of antibody labelled with horseradish peroxidase (HRP) and thus an increase in the chemiluminescent signal emitted. The light signal generated from each of the test regions on the biochip is detected using digital imaging technology and compared to that from a stored calibration curve. The concentration of analyte present in the sample is

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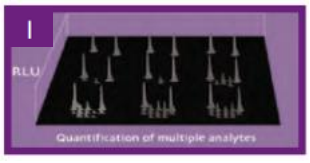
calculated from the calibration curve. Several different immunoassay based multi-analyte arrays have been developed for use on Evidence Investigator™.

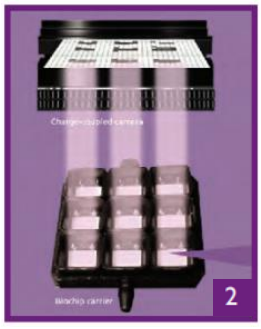
The Evidence Investigator™ Cytokine High-Sensitivity Array will quantitatively test for IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, IFNγ, EGF, MCP-1 and TNFI simultaneously.

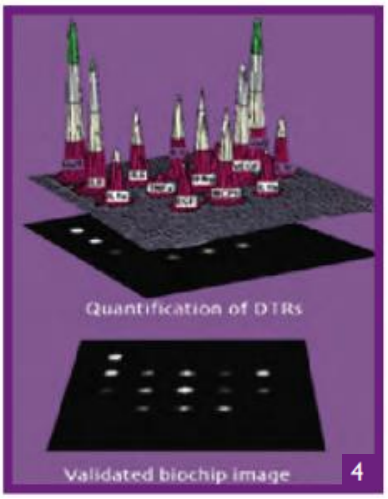
ARRAY DESCRIPTION:

INTERPRETATION OF THE RESULTS:

1. Unique imaging software on the analysers is used to translate the light signal into an analyte concentration.
2. The CCD Camera is made up of a collection of light sensitive diodes which convert light into electrons to produce an electrical charge. The brighter the light that hits a single diode, the greater the electrical charge that will accumulate at that site.







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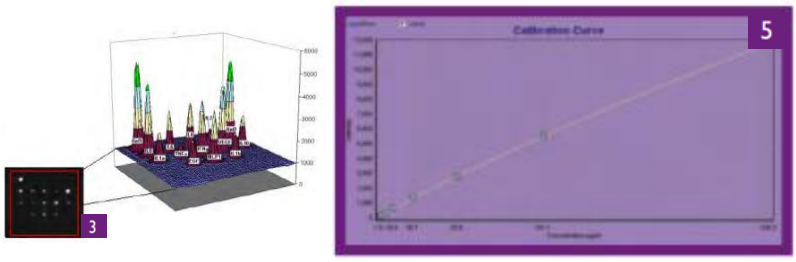
3. The degree of light emission can therefore be quantified based on the strength of the electrical signal generated.
4. A validated biochip image is then produced, which undergoes image analysis for quantification of the signal output at each DTR.
5. A validated calibration curve is used to determine the concentration of each analyte.



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4. Manual for CYTOKINE & GROWTH FACTORS HIGH SENSITIVITY ARRAY (CTK HS), Cat. No. EV 3623

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5. Computer Assisted Sperm Analysis - CASA

EQUIPMENT AND REAGENTS:

Equipment	
Equipment	Description
System	Sperm Vision® program
Light microscope	Olympus BX 51
Makler Counting Chamber	depth 10 µm
Micro pipettes	range from 0.5 µL to 1000 µL
Laboratory heating plate	
Reagent and Materials	
Deionized water	
Cotton pads	
Physiological solution	0.9% NaCl
Disposable gloves	
Tips	

METHOD DESCRIPTION:

The determination of the number of sperm that are moving rapidly in a forward progressive manner is of paramount importance in the evaluation of male fertility. In an average semen analysis, there are some spermatozoa with no movement, some that are moving slowly and/or some that do not move in a forward direction. These spermatozoa are less likely to be able to produce a pregnancy easily.

DESCRIPTION OF SPERMATOZOA MOVEMENT:

Illustration showing CASA terminology. Initial image processing provides a centroid for each spermatozoon in the first frame of a scene, and for each cell location of the most probable centroid in successive frames is deduced. Connecting the centroids for a spermatozoon provides its actual trajectory, termed curvilinear path. The time-averaged velocity along this trajectory is termed curvilinear velocity (VCL; µm/s). The average path is computed, and time-averaged velocity along this trajectory is termed average path velocity (VAP; µm/s). A straight-line path from the first to last position of a sperm head is plotted, and velocity along this trajectory is termed straight line velocity (VSL; µm/s). For each centroid location there is a deviation from the average path, and this is termed the amplitude of lateral head displacement (ALH; µm). Similarly, there are points where the curvilinear path intersects the average path, and the number of such intersections is termed beat-cross frequency (BCF; number per second). Sperm with rapidly progressive motility. They are strong and swim fast

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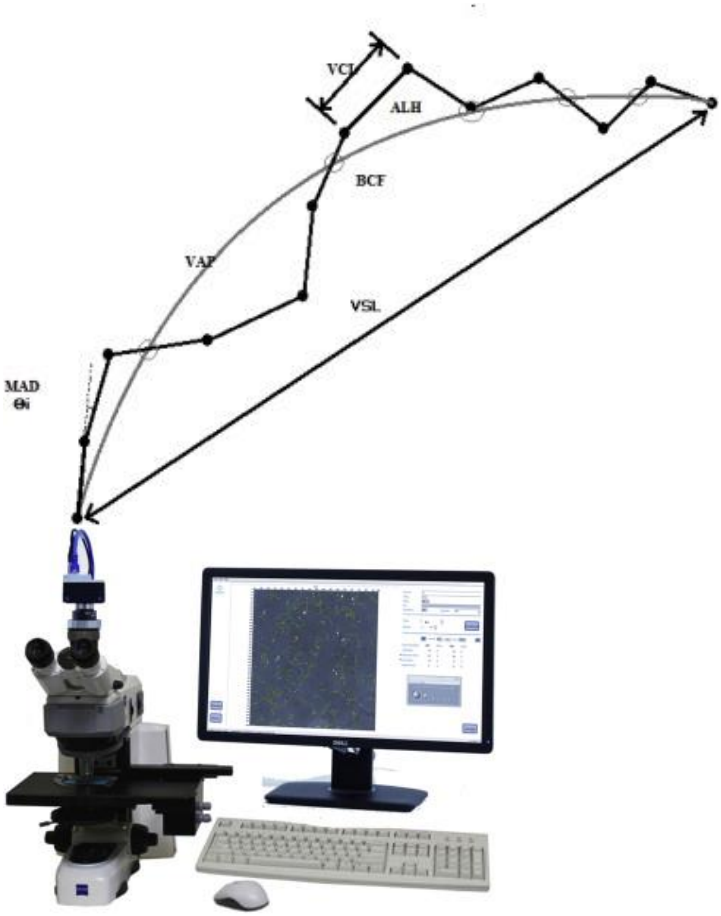
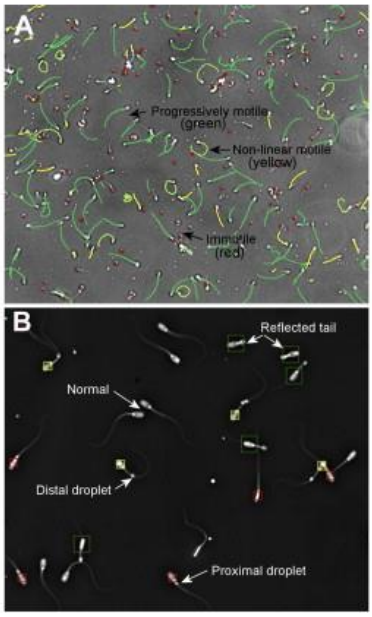
in a straight line, have progressive motility %, movements ≥25 µm/s. Motility, movements <5 µm/s.





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INTERPRETATION OF THE RESULTS:

Motion of each sperm is recorded as changes in centroid location in successive frames and computations provide output measures describing the motion (motility [PRO,%], progressive motility [PRO,%], curvilinear velocity [VCL, µm/s], average path velocity [VAP, µm/s], straight line velocity [VSL, µm/s], distance curved line [DCL, µm], distance straight line [DSL, µm], distance average path [DAP, µm], amplitude of lateral head displacement [ALH, µm], linearity of the curvilinear path [LIN], straightness of the average path [STR], and beat-cross frequency [BCF, Hz], wobble [WOB]). Within each of the CASA assessments, several different fields of view of Makler Counting Chamber are evaluate.

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6. Chemiluminescent reactive oxygen species determination

EQUIPMENT AND REAGENTS:

Equipment	
Equipment	Description
Luminometer	Glomax Multi^{+} (Promega Corporation; Madison,WI, USA)
Micro pipettes	Range from 2 µL to 1000 µL
Optional equipment	
Computer-aided semen analysis (CASA)	
Sperm counting chamber	
Reagent and Materials	
Dimethyl sulfoxide (DMSO)	Pure, 99%
Luminol	5-amino-2,3-dihydro-1,4-phthalazinedione; ≥97% (HPLC)
Hydrogen peroxide	30%
48-well plate	
Dulbecco's phosphate-buffered saline solution (DPBS)	
Disposable gloves	
Pipette tips	
1.5 mL tubes	
15 mL Falcon tube	

METHOD DESCRIPTION:

Principle

Luminol reacts with extracellular as well as intracellular free radicals and generates a light signal. Luminometer converts these signals to photons. The amount of oxygen free radicals is evaluated as relative light units (RLU)/s/10^6 cells.

Luminol solutions

One hundred mM stock solution (SS) of luminol is stable for approximately one month at room temperature, if not exposed to light. For this purpose, cover the Falcon tube with aluminium foil.

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Prepare the Working solution (WS) by mixing luminol SS and DMSO at a ratio of 1:15 in a 1.5 mL test tube. The solution is stable at room temperature for a maximum of 24 h, if not exposed to light.

ROS quantification

Samples put into 48-well plate as shown in the figure. Blank, negative as well as positive controls are put into the plate in triplicates. Make sure that the luminometer settings are ready to go before adding luminol into the microplate. When adding luminol, mix the well content properly, but avoid bubbles. Place the microplate into the luminometer and start the analysis immediately. When placing the microplate into the luminometer, be aware of the plate insertion setting to "48-well". The analysis includes 15 cycles of 1-min measurement. After 15 min., save the data to your flash drive.

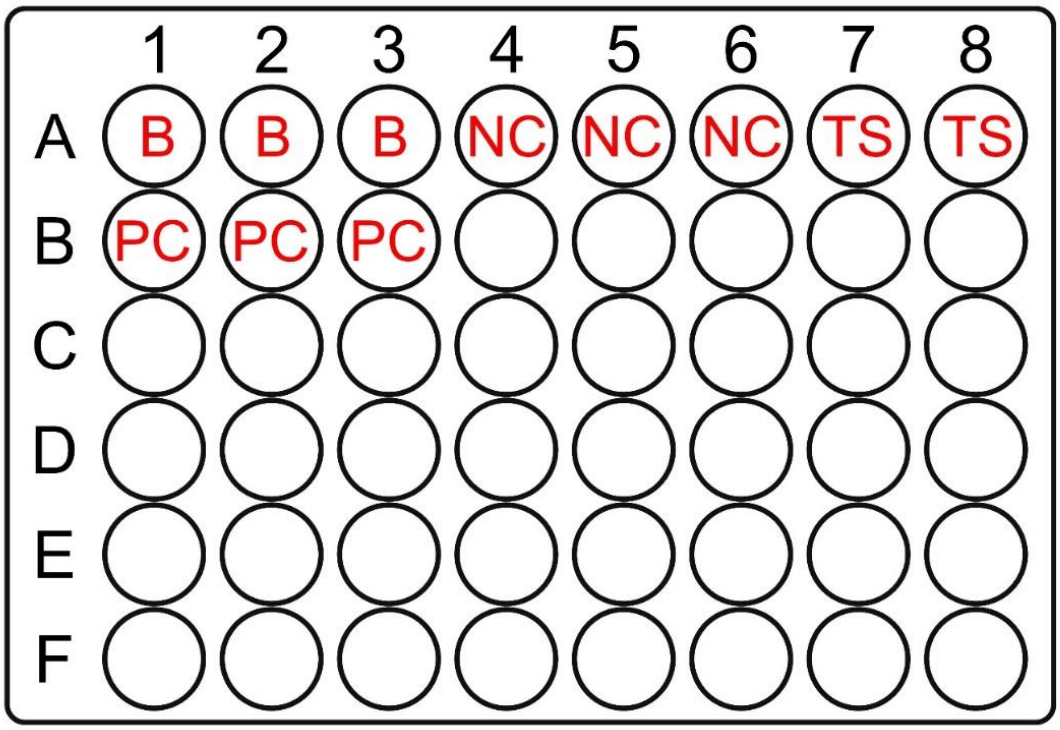

Figure 1: Setup design of 48-well microplate. **B** – blank: DPBS, **NC** – negative control: DPBS + luminol (WS), **TS** – tested sample: sample + luminol (WS), **PC** – positive control: DPBS + luminol (WS) + 30% H_2O_2.

Analysis of measured data

Open the excel file and calculate the average values for the controls and tested samples as well. Obtain the ROS value of tested samples by subtracting the NC average from the TS average. Divide the ROS value with "cell concentration/mL" value. Now, the result is indicated in RLU/s/10^6 cells.

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LITERATURE:

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7. Cell cultivation and cell passaging/subculturing

EQUIPMENT AND REAGENTS:

Equipment and reagents - not supplied with the kit	
Equipment	Description
Micropipettes	Range from 100 µL to 300 µL
CO2 incubator	Adjustable to 37 °C
Digital Mini Rotator	cat. no 88882007, Thermo Scientific
Laminar flow box	available
Optional equipment	
Equipment	Description
Multichannel Pipette	Range from 100 µL to 300 µL
Reagent and Materials	
HGL5 - human ovarian granullosa cells	
OVCAR-3 - human ovarian carcinoma cells	Disposable gloves
HT-29 -human colorectal adenocarcinoma cell line	Gibco Dulbecco's Modified Eagle Medium (DMEM)
H295R - human adrenal cortex-derived cell line	Medium 199 - M199
HUVEC - human umbilical vein endothelial cells	Antibiotics
Sterile filter tips	Trypsin
15- and 50-ml tubes	Dimethylsulfoxide (DMSO)
Multichannel Pipette Reservoir	ITS Premix – universal culture supplement
Nu-serum – growth medium supplements	Fetal bovine serum (FBS)
Endothelial Cell Growth Supplement (ECGS)	Hanks' Balanced Salt Solution (HBSS)
Cell culture flasks (T25, T75, T175, T225)	Heating water bath
96 well plate	PBS - phosphate buffered saline

METHOD DESCRIPTION:

Cell cultivation is conducted in culture medium under specific temperature and humidity conditions. Cells are cultivated either in suspension as individual cells or small groups of cells, or in the medium most often adhered on a suitable surface. The cultivation conditions should mimic as precisely as possible the in vivo physiological conditions provided by the culture

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medium and the technical equipment of laboratory (incubators). Culture medium consists of a mixture of several dozen substances. Medium contains a balanced ratio of amino acids, saccharides (a source of energy), vitamins, salts, lipids, trace elements, cofactors, pH stabilizers and inorganic salts. Also, growth factors have an important function in the medium. Blood serum, most commonly fetal bovine serum, is used as a source of growth factors, growth inhibitors, albumins, and other substances important for culture growth. The buffering component of the medium maintains a permanent pH which is very important for the cultivation conditions. To facilitate cell culture work, an acid-base indicator may be added to the medium that changes the color of the medium in the event of a change in pH. Sterility and stability of medium components are also a prerequisite for its use. Microbial contamination is a serious problem in cells cultivation process. To eliminate this phenomenon, a mixture of antibiotics - (1%) may be added to the culture medium. Cells are cultivated at body temperature in culture vessels and in the atmosphere with increased CO2 concentration (5-10%). After multiplication, the adherent cells are released from the culture surface (e.g., by using of proteases, mechanically or by changing the temperature) and the diluted suspension is pipetted into a new culture vessel - the so-called cells passaging. During cultivation, the number of cells increases exponentially in most cases until the moment the cells begin to touch tightly. As a result of contact inhibition, the growth of cells is then slowed down to stop. Most adherent cultures eventually create a layer on the surface of the cultivation vessel with a single cell thickness - a cell monolayer. Cultivated cells have a largely limited lifespan, after several passages they age, and their further growth gradually stops. Some cell types (some transformed or tumor-like cells) can be cultivated continuously.

ASSAY PROTOCOL: CELL PASSAGING/SUBCULTURING

1. Remove medium and trypsin from refrigerator and heat to 37 °C in a water bath.
2. Transfer the cultivation flask from incubator to the laminar flow box.
3. Using serological pipettes or a vacuum pump, carefully discard the medium from the culture flask so that we do not damage adhered cells.
4. Wash the cells with PBS (10 mL), which we also discard.
5. Pipette the trypsin (1 mL) into the culture flask, transfer it to the CO_2 incubator and let the enzyme act for about 3 minutes.
6. Return the culture bottle to the laminar box and quickly pipette the complete cultivation medium (10 ml) to stop the action of trypsin.
7. At this point, suspension of cells with cells released from the bottom of the flask is formed in the culture bottle. Several times we still thoroughly wash the bottom of the flask to get all the cells into cell suspension.
8. Transfer the cell suspension to a 50 mL conical tube, which we place in a centrifuge and centrifuge for 5 min. at 1200 rpm.
9. After centrifugation, transfer the tube back to the laminar box and carefully discard the supernatant so that only a clean cell pellet remains at the bottom of the tube.
10. To the pellet in the tube, we add a pure cultivation medium (10 mL), resuspend the pellet of cells in the medium thoroughly by pipette.

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11. Pipette a pure medium (15 mL) into a new, clean culture flask and then add a cell suspension (the volume of the cell suspension depends on the time of another passage planned).

LITERATURE:

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3. Michalcova, K., Roychoudhury, S., Halenar, M., Tvrda, E., Kovacikova, E., Vasicek, J., Chrenek, P., Baldovska, S., Sanislo, L., Kren, V. and Kolesarova, A. 2019. In vitro response of human ovarian cancer cells to dietary bioflavonoid isoquercitrin; Journal of environmental science and health. Part. B, Pesticides, food contaminants, and agricultural wastes, Vol. 54, No. 9, pp. 752–757.

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8. Cryopreservation of animal germ cells

EQUIPMENT AND REAGENTS:

Reagent and Materials	
Cryoprotective diluent	According to cell type and species
Plastic straws	250 µl
Manual straw filler	
Test tube	15 ml
Automated freezing box	IceCube
Freezing Styrofoam box	
Liquid nitrogen	
Disposable gloves	
Micropipets	

METHOD DESCRIPTION:

Sperm cryopreservation (Ram)

Semen samples are diluted with a Triladyl® (commercial diluent containing glycerol and antibiotics (Minitube, Tiefenbach, Germany) and supplemented with egg yolk immediately before use) at the ratio of 1:10 (v/v). Afterward, sperm samples are loaded into straws (250 µl) and then equilibrated at 4°C in a fridge.

Slow freezing by a programmable device

The semen straws are transferred into a pre-cooled automated freezing box (IceCube, Minitube, Germany). The lid is closed immediately and the calculated volume of nitrogen vapour is poured according to the freezing program (+4°C, −10°C (2 min), −80°C (7.5 min), −120°C (1.7 min), −140°C (3 min).

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Manual freezing in a freezing box

The semen straws are transferred on the floating rack into the freezing Styrofoam box (Minitube, Germany) with LN_2. The straws are suspended horizontally in LN_2 vapours 5 cm above the LN_2 level for 10 min (−125 to −130°C) and then straws were plunged into LN_2 (−196°C).

Oocyte cryopreservation (Bovine)

Matured oocytes are placed in 0.5 ml of equilibration solution (7.5% DMSO, 7.5% EG) for 10 min, then transfered to 0.5 ml of freezing solution (15% DMSO, 15% EG). The samples are loaded into straws and transferred into an automated freezing box (IceCube, Minitube, Germany). The lid is closed immediately and the calculated volume of nitrogen vapour is poured according to the freezing program (+20°C, −7°C (2°C/min), seeding- maintaining the temperature at 7°C for 10 min, −30°C (0.3°C/min), −150°C (50°C/min).

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INTERPRETATION OF THE RESULTS:

The results of performing this method are samples of biological material of selected endangered or otherwise important animal species. Cryopreserved samples are capable of storage in liquid nitrogen for decades. Samples treated and stored in this way are intended for the purposes of biodiversity protection as well as for research in many areas of biology (biotechnology, reproduction, cell biology, etc.). Another task of cryopreservation is to increase the effectiveness of artificial insemination (more efficient handling, storing and transport of insemination doses).

LITERATURE:

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9. DNA fragmentation with the apo-direct assay kit

EQUIPMENT AND REAGENTS:

Equipment and reagents - not supplied with the kit	
Equipment	Description
96-well Elisa Plate Reader	Cat. no 51119000, Multiscan
Thermostat or heating block	Adjustable to 37 °C
Vortex mixer	Cat. No BS-VOR-P, Benchmark Scientific
Fluorometer	Cat. No E7051, Promega Corporation
Micro pipettes	Range from 0.50 µL to 200 µL
Centrifuge	With cooling
Reagent and Materials	
Samples	Cells fixed in paraformaldehyde and washed in 70% ethanol
Apo-Direct Apoptosis Detection Kit	Cat. No 88-6611-88, Invitrogen
2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI)	1 µM in PBS Cat. No D9542, Sigma Aldrich
Distilled water	
Disposable gloves	
Eppendorf tubes	
Sterile tips	
Ice	
96 well plate	Black

METHOD DESCRIPTION:

One of the most easily measured features of apoptotic cells is the break-up of the genomic DNA by cellular nucleases. These DNA fragments can be extracted from apoptotic cells and result in the appearance of DNA laddering when the DNA is analyzed by agarose gel electrophoresis. The DNA of non-apoptotic cells that remains largely intact does not display this laddering on agarose gels during electrophoresis. The large number of DNA fragments appearing in apoptotic cells results in a multitude of 3'-hydroxyl termini in the DNA. This property can be used to identify apoptotic cells by labeling the 3'-hydroxyl ends with directly conjugated fluorescein- deoxyuridine triphosphate nucleotides (FITC-dUTP). The enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes a template-independent addition of deoxyribonucleoside triphosphates to the 3'-hydroxyl ends of double- or single- stranded DNA with either blunt, recessed, or overhanging ends. A substantial number of these sites are available in apoptotic cells providing the basis for the method utilized in the TUNEL assay.

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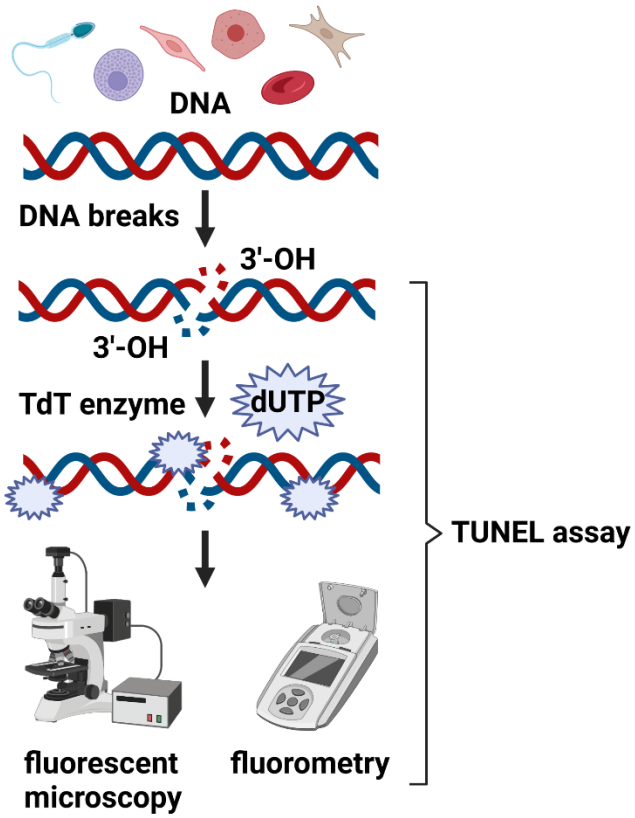
Non-apoptotic cells do not incorporate significant amounts of the FITC-dUTP due to the lack of exposed 3'-hydroxyl DNA ends.

The Apo-Direct TUNEL Assay Kit is a 2-color staining method for labeling DNA breaks and total cellular DNA to detect apoptotic cells by fluorometry. The kit contains the instructions and reagents required for measuring apoptosis in cells including positive and negative control cells for assessing reagent performance; washing, reaction and rinsing buffers for processing individual steps in the assay; terminal deoxynucleotidyl transferase enzyme (TdT), fluorescein-deoxyuridine triphosphate (FITC) and propidium iodide (PI) solution.



Principle of the technique

ASSAY DESCRIPTION:

1. Resuspend the positive and negative control cells by swirling the vials. Remove 1 mL aliquots of the control cell suspensions (approximately 1x10^6 cells per 1 mL) and place in centrifuge tubes. Centrifuge the control cell suspensions for 5 minutes at 300 x g, then remove the 70% (v/v) ethanol by aspiration, being careful to not disturb the cell pellet.

2. Resuspend each tube of control cells with 1 mL of Wash Buffer. Centrifuge as before and remove the supernatant by aspiration.

3. Repeat the Wash Buffer treatment.

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4. Resuspend each tube of the control cell pellets in 50 µL of the DNA Labeling Solution (prepared as described below):

DNA LABELING SOLUTION	1 ASSAY
TdT Reaction Buffer	10.00 µL
TdT Enzyme	0.75 µL
Fluorescein-dUTP	8.00 µL
Distilled H_2O	32.00 µL
Total Volume	51.75 µL

5. Incubate the cells in the DNA Labeling Solution for 60 minutes at 37°C in a temperature-controlled bath. Shake cells every 15 minutes to resuspend.

6. At the end of the incubation time add 1.0 mL of Rinse Buffer to each tube and centrifuge each tube for 5 minutes at 300 x g. Remove the supernatant by aspiration.

7. Repeat the cell rinsing as in step 6 and remove the supernatant by aspiration.

8. Resuspend the cell pellet in 0.5 mL of the Propidium Iodide solution. Add 10 µL DAPI to stain the cell nuclei.

9. Incubate the cells in the dark for 30 minutes at room temperature.

10. Transfer the content of the tubes into the black 96-well plate.

11. Analyze the samples by fluorometry within 3 hours of staining.

INTERPRETATION OF THE RESULTS:

1. Subtract the negative control from your assay reading. This is the corrected fluorescence.
2. The amount of DAPI fluorescence (blue) is proportional to cell number (100%).
3. The amount of FITC fluorescence (green) is proportional to the percentage of cells depicting damaged DNA.
4. The amount of PI fluorescence (red) is proportional to the percentage of necrotic cells.

LITERATURE:

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10. Eosin-Nigrosin Staining

EQUIPMENT AND REAGENTS:

Equipment and reagents - not supplied with the kit	
Equipment	Description
Micro pipette	2-20 µL
Light microscope	10x/22 x 40
Reagent and Materials	
Eosin Y-1% solution	
Nigrosin 10% solution	
Microscopic slides	
Tips	

METHOD DESCRIPTION:

The test consists of two main procedure steps.

- Preparation of samples
- Microscopical evaluation of samples

Preparation of samples

Prior to the sample preparation, all slides need to be labeled with the number and origin of the specimen. 3µl of ejaculate (diluted with physiological solution to approx. concentration of <20 x10^6) is pipetted on the left end of the microscopic slide. Immediately after that, 6 µL of 1 % aqueous Eosin Y is placed directly in the drop of the ejaculate and the specimen with the dye are thoroughly mixed in the tip and released back on the microscopic slide. After at least 15 seconds, 6 µL of Nigrosin is added to the mixture of specimen and Eosin and all the liquids are well mixed in the micro pipette tip. Following, arose mixture is smeared across the whole microscopic slide with the use of another microscopic slide applied under 45° angle. Afterwards, smears of the spermatozoa stained with Eosin-Nigrosin mixture are left to slowly dry out on the air.

Microscopical evaluation of samples

The smears are evaluate using brightfield microscopy (10x/22 x 40; KRÜSS MBL3200, A. KRÜSS Optronic GmbH, Hamburg, Germany). The number of at least of 100 randomly selected spermatozoa are evaluated based on the coloring of their heads. The examiner needs to

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distinguish between the white to faint pink heads and red to dark pink colored heads and mark down the number of events within each group.

INTERPRETATION OF THE RESULTS:

Eosin is responsible for the staining of the dead spermatozoa while the role of the Nigrosin is to enhance the color contrast between stained heads and the background. Eosin stains only the heads of the spermatozoa with damaged membrane which are thus classified as dead. Heads of dead spermatozoa have red to dark pink coloring. Spermatozoa with intact membrane, therefore considered live, have their heads colored in white or light pink. The ratio between live and dead spermatozoa is carried out based on the total number of evaluated spermatozoa within the sample and is interpreted in percentage.

LITERATURE:

1) Agarwal A., Gupta S. and Sharma R. 2016. Eosin-Nigrosin Staining Procedure. In: Agarwal A., Gupta S., Sharma R. (eds) Andrological Evaluation of Male Infertility. Springer, Cham. https://doi.org/10.1007/978-3-319-26797-5_8
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11. Enzyme-linked immunoassay protocol

EQUIPMENT AND REAGENTS:

Equipment and reagents - not supplied with the kit	
Equipment	Description
96-well Elisa Plate Reader	cat. no 51119000, Multiscan
CO2 incubator	Adjustable to 37 °C
Digital Mini Rotator	cat. no 88882007, Thermo Scientific
Micro pipettes	Range from 100 µL to 300 µL
Optional equipment	
Equipment	Description
Multichannel Pipette	Range from 100 µL to 300 µL
Reagent and Materials	
Samples	
ELISA kit	
Distilled or deionized water	
96 well plate	
Disposable gloves	
Sterile filter tips	
15 and 50 ml tubes	
Multichannel Pipette Reservoir	

METHOD DESCRIPTION:

ELISA (which stands for enzyme-linked immunosorbent assay) is a technique to detect the presence of antigens in biological samples. An ELISA, like other types of immunoassays, relies on antibodies to detect a target antigen using highly specific antibody-antigen interactions. In an ELISA assay, the antigen is immobilized to a solid surface. This is done either directly or via the use of a capture antibody itself immobilized on the surface. The antigen is then complexed to a detection antibody conjugated with a molecule amenable for detection such as an enzyme or a fluorophore. An ELISA assay is typically performed in a multi-well plate (96- or 384-wells), which provides the solid surface to immobilize the antigen. Immobilization of the analytes facilitates the separation of the antigen from the rest of the components in the sample. This characteristic makes ELISA one of the easiest assays to perform on multiple samples simultaneously.

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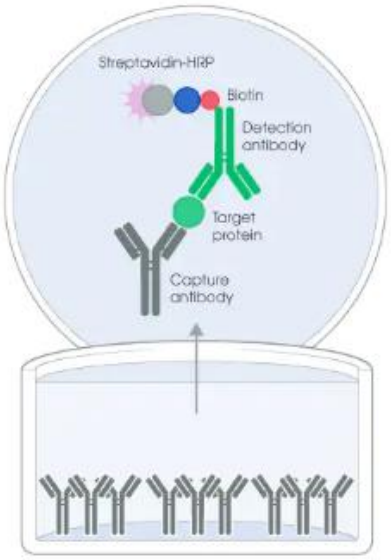

The basic setup of an ELISA assay. A capture antibody on a multi-well plate will immobilize the antigen of interest. This antigen will be recognized and bound by a detection antibody conjugated to biotin and streptavidin-HRP.

ASSAY PROTOCOL:

1. Prepare Coating Solution by diluting the Capture antibody in Coating buffer. Refer to manufacturer for dilution recommendations.
2. Coat plates with 100 µL per well of Coating Solution. Cover plates, and incubate overnight (12–18 hours) at 2–8 °C.
3. Aspirate wells and wash 1 time with >200 µL of Wash buffer per well. Following wash, invert and tap on absorbent paper to remove excess liquid.
4. Block plate with 200 µL per well with Blocking buffer for 1 hour at room temperature.
5. Aspirate, invert, and tap on absorbent paper to remove excess liquid.
6. Prepare standards and sample dilutions in Blocking buffer.
7. Pipette 100 µL of standards (in duplicate) and samples into designated wells. Incubate for 1 hour at room temperature with gentle continual shaking (~500 rpm).
8. Aspirate and wash 5 times with >200 µL of Wash buffer per well. Following wash, invert and tap on absorbent paper to remove excess liquid.
9. Prepare detection antibody solution by diluting the Detection antibody in Blocking buffer. For recommended antibody dilution, refer to manufacturer's instruction.
10. Add 100 µL of the detection antibody solution into each well. Incubate for 2 hours at room temperature with gentle continual shaking (~500 rpm).
11. Aspirate and wash 5 times with >200 µL of Wash buffer per well. Following wash, invert and tap on absorbent paper to remove excess liquid.

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12. Make working solution of Streptavidin-HRP with Blocking buffer by diluting 1:5,000. For example, to make enough for 1 plate, add 2 µL of streptavidin-HRP to 9.998 mL of Blocking buffer.
13. Add 100 µL of working streptavidin-HRP solution into each well. Incubate for 30 minutes at room temperature with gentle continual shaking (~500 rpm).
14. Aspirate and wash 5 times with >200 µL of Wash buffer per well. Following wash, invert and tap on absorbent paper to remove excess liquid.
15. Add 100 µL of TMB substrate solution to each well. Incubate plate for 30 minutes at room temperature.
16. Add 100 µL of Stop solution to each well.
17. Measure absorbance at 450 nm within 30 minutes of adding Stop solution.

DATA ANALYSIS:

1. Average the duplicate reading for each sample.
2. Calculate results using a log-log or 4-parameter curve fit.

LITERATURE:

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2) Abcam. 2021. ELISA principles and types. https://www.abcam.com/kits/elisa-principle
3) Thermo Fisher. 2021. General Sandwich ELISA Protocol. https://www.thermofisher.com/sk/en/home/references/protocols/cell-and-tissue-analysis/elisa-protocol/general-elisa-protocol.html?ef_id=Cj0KCQiAtJeNBhCVARIsANJUJ2Hz0alBBCI8kKojrVcncOOwxGGABrb1vUjwJNefVOaCAHZY-xH-J-QaAkZXEALw_wcB:G:s&s_kwcid=AL!3652!3!515826766954!e!!g!!elisa%20protocol&cid=bid_pca_iel_r01_co_cp1359_pjt0000_bid00000_0se_gaw_nt_pur_con&gclid=Cj0KCQiAtJeNBhCVARIsANJUJ2Hz0alBBCI8kKojrVcncOOwxGGABrb1vUjwJNefVOaCAHZY-xH-J-QaAkZXEALw_wcB

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12. Ferric-reducing ability of plasma (FRAP)

EQUIPMENT AND REAGENTS:

Equipment and reagents - not supplied with the kit	
Equipment	Description
Spectrophotometer	Adjustable to 593 nm
Cuvettes	
Thermostat or heating block	Adjustable to 37 °C
Micropipettes	Range from 10 µL to 1000 µL
Graduated cylinders	Range from 10 mL to 100 mL
Reagent and Materials	
Sodium acetate	Cat. No. S2889, Sigma Aldrich
Acetic acid	Cat. No. A6283, Sigma Aldrich
2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ)	Cat. No. T1253, Sigma Aldrich
Hydrochloric acid (HCl)	Cat. No. H1758, Sigma Aldrich
Iron(III) chloride hexahydrate ($FeCl_3.6H_2O$)	Cat. No. 236489, Sigma Aldrich
Ascorbic acid	Cat. No. A92902, Sigma Aldrich
Distilled water	
Disposable gloves	
Pipette tips	

METHOD DESCRIPTION:

The ferric reducing antioxidant power (FRAP) mechanism is based on electron transfer rather than hydrogen atom transfer. The FRAP assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+}. The FRAP reaction is conducted at acidic pH 3.6 to maintain iron solubility, so the reaction at low pH decreases the ionization potential that drives hydrogen atom transfer and increases the redox potential, which is the dominant reaction mechanism. When the reduction of Fe^{3+} to Fe^{2+} occurs in the presence of 2,4,6-trypyridyl-s-triazine, the reaction is accompanied by the formation of a colored complex with Fe^{2+} (absorption at 593 nm). The reducing power appears to be related to the degree of hydroxylation and extent of conjugation in antioxidants. Because the reaction detects compounds with redox potentials of <700 mV, which is comparable with that of ABTS•+ (680 mV), similar compounds react in both the TEAC and FRAP assays. FRAP cannot detect compounds that act by radical quenching (hydrogen transfer), particularly thiols (as glutathione) and proteins. However, FRAP is simple, rapid (generally 4–6 min), inexpensive and can be performed using semiautomatic or automated protocols.

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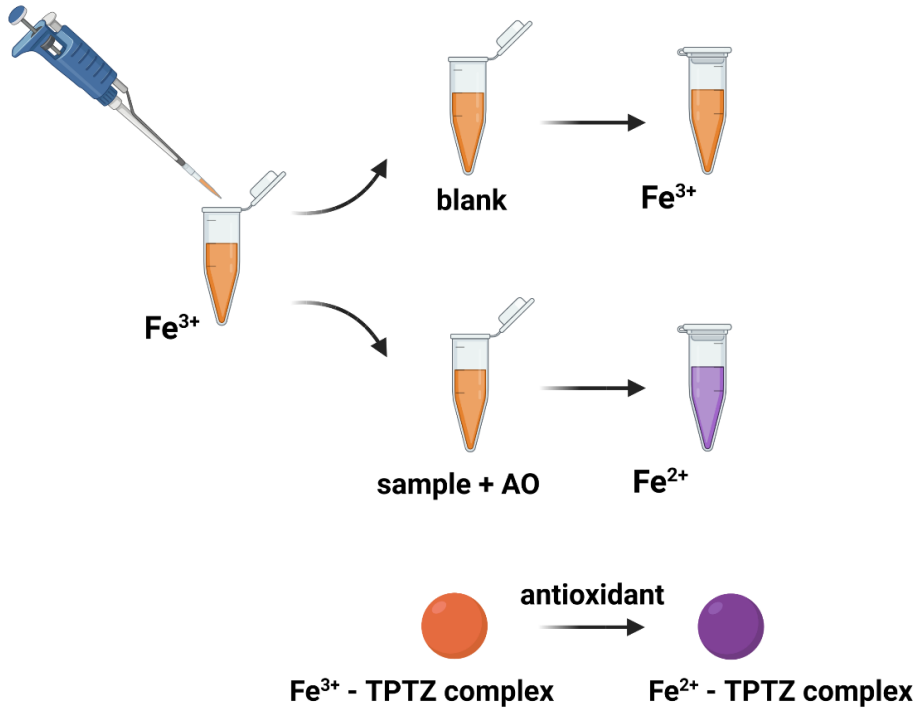

Principle of the method

ARRAY DESCRIPTION:

1. Prepare the reagents:

Reagent A – 300 mmol/L acetate buffer

Reagent B – 10 mmol/L TPTZ in 40 mmol/L HCl

Reagent C – 20 mmol/L $FeCl_3.6H_2O$

Mix the reagents A, B and C in a ratio of 10 (A) : 1 (B) : 1 (C)

2. Prepare the standard: 1000 µmol/L ascorbic acid in distilled H_2O

3. Mix 10 µL standard or sample with 300 µL of the FRAP reagent. Measure absorbance 1 (A1) at 593 nm immediately.

4. Incubate the sample at 37 °C for 4 minutes. Measure absorbance 2 (A2) at 593 nm.

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INTERPRETATION OF THE RESULTS:

1. Obtain the change in absorbance (ΔA_{593nm}) between A2 and A1

2. Calculation:

FRAP (µmol/L) = ($\Delta A_{sample} \div \Delta A_{standard}$) x concentration of the standard

LITERATURE:

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3. Pahune, P.P., Choudhari, A.R., and Muley, P.A. 2013. The total antioxidant power of semen and its correlation with the fertility potential of human male subjects. Journal of clinical and diagnostic research; Journal of Clinical and Diagnostic Research, Vol. 7, No. 6, pp. 991–995.

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13. Hematology

EQUIPMENT AND REAGENTS:

Optional equipment	
Equipment	Description
Abacus Vet5 Hematology Analyzer AV5	Abacus Vet5 software v1.1; DIATRON MI ZRT, Budapest, Hungary
Reagent and Materials	
animal blood sample	
EDTA blood collection tubes	
Junior VET-5 part pack + HK	DRP02HK

METHOD DESCRIPTION:

Abacus Vet5 hematology analyzer is a quantitative, fully automated, multi-parameter, bench-top cell counter designed for *in vitro*-diagnostic use in veterinary clinics, research laboratories and point-of-care centers for enumeration of 24 hematology parameters. It provides precise and accurate 5-part (or in certain cases 3-part) WBC differential measurement results using impedance measurement technology.

Abacus Vet5 analyzer uses the volumetric impedance method for measuring leukocytes (WBC), erythrocytes (RBC) and platelet (PLT) concentrations. Measurement of the hemoglobin (HGB) content of red blood cells is accomplished by photometric measurement technology. 5-part WBC differential accomplished by using a differential lysing reagent.

ASSAY DESCRIPTION:

Depending on the working mode the analyzer takes 25 or 50µl of K_2 or K_3-EDTA anti-coagulated whole blood from an open sample vial placed in the sample rotor station. It can process 24-30 samples per hour in 3-part mode, 16-20 samples per hour in 5-part mode.

Abacus Vet5 analyzer uses a combination of methods to determine the measurement results:

- Volumetric impedance method is used to determine the cellular concentrations and volume distributions of erythrocytes (RBC), and platelets (PLT) from so-called RBC dilution.
- Volumetric impedance method is used in combination with a lytic reagent to determine distribution of leukocytes (WBC), and their three sub-populations (LYM, MON, NEU, EOS, BAS).

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- Photometric measurement of light absorbance of the WBC dilution determines hemoglobin (HGB) concentrations of erythrocytes (RBC).

MEASURED PARAMETERS:

Parameter	Description	Reported Unit	Calculation method
WBC	White Blood Cell Count	cells/l, cells/µl	Number of leukocytes WBC = WBCcal x counted WBC
RBC	Red Blood Cell Count	cells/l, cells/µl	Number of erythrocytes RBC = RBCcal x counted RBC
HGB	Hemoglobin concentration	g/dl, g/l, mmol/l	Measured photometric way at 540 nm; in each cycle blank measurement is performed on diluent. HGB = HGBcal x (HGBmeasured - HGBblank)
MCV	Mean Corpuscular Volume	fl	Average volume of individual erythrocytes derived from the RBC histogram.
HCT	Hematocrit	percentage, absolute	Calculated from the RBC and MCV values. HCTpercentage = HGB x MCV / 10 HCTabsolute = HCTpercentage / 100
MCH	Mean Corpuscular Hemoglobin	pg, fmol depends on HGB unit selected	Average hemoglobin content of erythrocytes, calculated from RBC and HGB values. MCH = HGB / RBC
MCHC	Mean Corpuscular Hemoglobin Concentration	g/dl, g/l, mmol/l equals to HGB unit selected	Calculated from the HGB and HCT values. MCHC = HGB / HCTabsolute
RDW-SW	Red Cell Distribution Width – SD	fl	Distribution width of the erythrocytes or platelet population derived from the histogram at 20% of peak xDW-SD = RDWcal x (P2- P) (fl) xDW-CV = RDW x 0.56 x (P2 – P1) / (P2 + P1) by the factor of 0.56 CV is corrected to the 60% cut
PDW-SW	Platelet Distribution Width – SD	fl	
RDW-CV	Red Cell Distribution Width – CD	%	
PDW-CV	Platelet Distribution Width – CV	%	
PLT	Platelet count	cells/l, cells/µl	Number of thrombocythes (platelets) PLT = PLTcal x counted PLT
PCT	Thrombocrit	percentage, absolute	Caltulated from the PLT and MPV values PCTpercentage = PLT x MPV / 10.000

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			PCTabsolute = PLTpercentage / 100
MPV	Mean Platelet Volume	fl	Mean volume of platelets derived from PLT histogram MPV has its own calibration factor.
LYM, LYM%	Count and % of lymphocyte cells	cells/l, cells/µl and %	Absolute values counted in the channels determined by the three WBC discriminators. Percentages calculated from the absolute WBC value. GRA = NEU + EOS + BAS
MON, MON%	Count and % of medium size cells	cells/l, cells/µl and %	
NEU, NEU%	Count and % of neutrophil cells	cells/l, cells/µl and %	
BAS, BAS%	Count and % of basophil cells	cells/l, cells/µl and %	
EOS, EOS%	Count and % of eosinophil cells	cells/l, cells/µl and %	

INTERPRETATION OF THE RESULTS:

White Blood Cells and Associated Indications

White Blood Cell (Leukocyte)	Role	Increase in Disease State	Decrease in Disease State
Non-Granulocytic			
Lymphocytes	**B-cells:** humoral immunity (antibody synthesis) **T-cells:** cellular immunity	Chronic inflammation Acute infection/recovery Lymphocytic leukemia Hypoadrenocorticism	Acute/severe disease Viral disease Endotoxemia Hyperadrenocorticism Stress-related corticosteroid response
Monocytes	Immature macrophages – phagocytosis of debris/foreign material, killer-cell activation	Necrotic, malignant, hemorrhagic, or immune-mediated disease	Rare, no know significance
Granulocytes			
Neutrophils	Phagocytize/kill	Inflammation Neoplasm	Bacterial infection Viral Infection

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Left shift: Increased Numbers of immature neutrophils/band cells. - regenerative: up to 50% bands, neutrophilia, absence of myelocytes / metamyelocytes - degenerative: > 10% bands, depressed total neutropenia, presence of myelocytes/metamyelocytes (poor prognostic indicator) ***Right shift:*** Increased Number of hypermature (hyper-segmented) neutrophils, often seen with non-infectious inflammatory (e.g. malignancy)	microorganisms, initiate and modify Inflammatory process, cytotoxic	Stress Exercise/excitement	Drug-induced (bone-marrow depression)
Eosinophils	Parasiticidal Cytotoxic Phagocytic	Parasitic infection Allergic responses Hypoadrenocorticism	Stress Hyperadrenocorticism ACTH therapy
Basophils	Initiate inflammation Prevent coagulation Activate lipoprotein lipase	Allergic reactions Parasitic infections Neoplasia	No know significance

Red Blood Cell Parameters and Associated Indications

Parameter	Definition	Diagnostic Consideration
Hematocrit (HCT)	Percentage of total cellular constituents (primarily red blood cells) in a unit of whole blood	Anemia exists when the HCT falls below the reference range for the species. Hematocrit will normally have a value of

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		approximately three times the hemoglobin value
Hemoglobin (HGB)	The oxygen-carrying component of red blood cells; allows for the calculation of MCH and MCHC	Hemoglobin normally falls in the range of one third of the hematocrit value.
RBC Indices Anemia Characterization		
MCV Mean Corpuscular Volume	Measure of the volume of an average RBC	Increase: most commonly associated with reticulocytes / regenerative anemia Decrease: iron-deficiency anemia Normal MCV is consistent with non-regenerative anemia, often due to chronic disease. MCV should always be interpreted in light of other clinical data.
MCH Mean Corpuscular Hemoglobin	Calculated HGB concentration of an average RBC (pg)	Increase: most commonly the result of hemolysis Decrease: hypochromasia common in iron-deficiency anemia and reticulocytosis
MCHC Mean Corpuscular Hemoglobin Concentration	Calculated HGB concentration of an average RBC (g of HGB per 100 ml RBCs)	In the anemic state, normal MCHC (with normal MCV) is consident with non-regenerative anemia due to chronic disease. Decrease: hypochromasia common in iron-deficiency anemia and reticulocytosis.
RDW Red Cell Distribution Width	Measure of red blood cell anisocytosis (cell size variation)	Elevated RDW is typically indicative of anisocytosis. In the anemic state, increased RDW with an associated increase in MCV can indicate increased levels of immature RBCs.

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Platelet Parameters and Associated Indications

Parameter	Increase in Disease State	Decrease in Disease State
Total Platelet Count	Thrombocytosis is present with excess bleending, iron deficiency anemia and myeloproliferative syndromes.	Disseminated intravascular coagulation Bone marrow depression Autoimmune hemolytic anemia Severe hemorrhage Liver disease Parasites
MPV Mean Platelet Volume	Indirect evidence of increased (bone marrow) megakaryocyte response.	Not an accurate predictor of decreased megakaryocyte response.
PCT Platelet hematocrit	Volume of platelets expressed as a percentage of whole blood (used as a research tool).	Volume of platelets expressed as a percentage of whole blood (used as a research tool).
PDW Platelet Distribution Width	Increased measure of platelet anisocytosis (platelet size variation) indicative of active platelet release.	No know clinical significance.

LITERATURE:

1. Abacus Vet5 User´s Manual, DIATRON MI ZRT, Táblás utca 39, Budapest, Hungary.

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14. Identification of bacteria using MALDI-TOF

EQUIPMENT AND REAGENTS:

Equipment	
Equipment	Description
Biological Safety Cabinet	Best choice Biological Safety Cabinet Class II or higher (any manufacturer) with gas connection, HEPA filters, electricity.
MALDI-ToF MS Bruker Biotyper	Minimal requirements: Microflex LT from Bruker Daltonics with Biotyper software and available spectral database of microorganisms. Software requirements: Biotyper, flexControl
Pipettes	Range from 1 µl to 1000 µl
Reagent and Materials	
100% Ethanol-Sterile Solution	SIGMA or equivalent
Sterile WFI Quality Cell Culture Grade Water	Fisher Scientific or equivalent
Growing media by tested bacterial groups	Growing media is depended on cultivated bacterial strain (any manufacturer)
Plates and slants	Fisher Scientific or equivalent
Reusable polished steel MALDI target plate with 96 sample positions	Bruker Daltonics (Stainless steel polished 96-plate target)
Bruker matrix HCCA (HCCA = α-Cyano-4-hydroxycinnamic acid)	SIGMA (Cat. #C8982) or equivalent
70% Formic Acid	SIGMA (Cat. #00940) or equivalent
Acetonitrile suitable for mass spectrometry	SIGMA (Cat. #34851) or equivalent
TFA – trifluoroacetic acid suitable for HPLC ≥ 99.0%	SIGMA (Cat. #302031)or equivalent
Bacterial test standard (BTS)	Bruker Daltonics (*E. coli* DHB alpha extract) - standard
Bruker Standard Solvent	Sigma Aldrich or can be prepared by standard bruker procedure
1µl Culture loops	Any manufacturer (metal or plastic)
Gloves	Standard nitrile gloves (any manufacturer)
Sterile filter tips	1mL, 100µL, 1 µL
Microtubes	1.5ml microtubes (Ependorf tubes)

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METHOD DESCRIPTION:

The procedure for identification of bacterial isolates using the MALDI-ToF MS Bruker Biotyper platform and a custom extraction protocol optimized to identify isolates that are represented in the Bruker.

ASSAY PROTOCOL:

1. **Sample Preparation Information/Processing**

Growth

- Overnight growth of a pure bacterial culture should be used for routine microorganism identification;
- Slow-growing bacterial strain (cultivated more then 24 hours) may need to grow for several days before testing.

Storage

- Store plates at room temperature for 1-5 days is acceptable;
- Culture medium and growth temperature (25°C vs 37°C) have little to no effect on results.

Quality Control

- For each run, include pure culture of *E. coli* (Bruker daltonics bacterial test - BTS), in duplicate, as positive controls.

2. ***Preparation of organic solvent for HCCA matrix***

- Create mixture of 50 % acetonitrile + 47,5 % sterile purified water + 2,5 % TFA (trifluoracetic acid) to amount of 250 µL.
- Add one full loop of HCCA matrix powder. Ensure that the solution is supersaturated.

3. ***Extraction Procedure***

- Perform extraction procedure in duplicate for better results of identification.
- Create 60 % ethanol solution by mixing of 100 % ethanol and high water purity.
- Label two 1.5 mL microcentrifuge tubes for each sample, including BTS strains.
- Add form 50-100 µL of 60 % ethanol to each tube independent on bacterial amount.
- Using a sterile, clear 1 µl inoculating loop, transfer 1 loopful of microorganism to both tubes containing 60 % ethanol. If the colonies are small, transfer more than one colony, choosing isolated colonies, when possible.
- Secure the lid of the tube.

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- Using a 100 µl pipette set to 50 µl, pipette the liquid up and down 10 times to homogenize or use homogenizator.
- Insert tubes to centrifuge and centrifuge at 14 000 rpm.
- After centrifugation discard the whole ethanol from the tube with taking into account the preservation of the microorganisms pellet.
- Add 20-50 µl formic acid (FA) and mix the pellet for membrane desintegration (amount of FA is depended on bacterial colonies).
- Add 20-50 ul acetonitrile (amount is a same like FA) for protein extraction.
- Cretaed solution should be centrifuged at 14 000 rpm for 2 minutes.
- After centrifugation, remove 1 µl of supernatant from each tube and transfer to a 96-spot reusable stainless steel target plate (see Fig. 1).

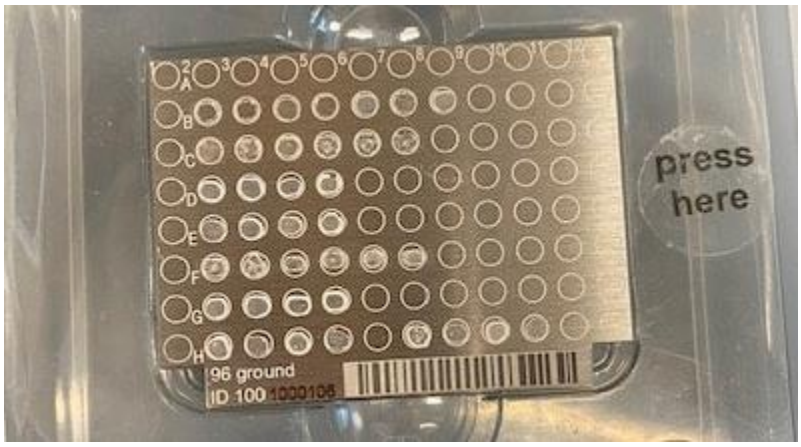

Fig. Bruker Daltonics Stainlees steel target plate

- Let the plate air dry to complet drying.
- Add 1 µL of the BTS suspension to two open spots on the 96-spot reusable stainless steel target plate
- As soon as spots are dry, immediately overlay with 1 µL of HCCA matrix (see preparation of HCCA matrix).
- Air dry for two minutes.
- As soon as spots are dry, they are ready to be subject to MALDI-TOF analysis.
- Once the target plate is dry, the plate must be run within 24 hours.
- Be sure that the location of each specimen on the 96-spot reusable stainless steel target plate is recorded on a sample key spreadsheet. Verify the spreadsheet numbers to make sure they match the original sample numbers.
- Samples are now ready for the MALDI-ToF MS assay on the Bruker Biotyper platform.
- Resulting spectra must be interpreted using Bruker database.

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INTERPRETATION OF THE RESULTS:

- Review control strains.
  - At least one of the duplicates for each control strain must generate a score of ≥ 2 with a correct identification (no conflicting results between the duplicates).
  - If the control strains generate scores < 2 or incorrect identification, the results cannot be interpreted, and the extraction/submission process must be repeated.
- Bacterial species will be identified if database match scores are ≥ 2 for one or more of the duplicates.
- If no species identification is made, repeat extraction/submission process using a new subculture.
- If no identification is successful after second submission, the isolate must be identified by another methodology.

LITERATURE:

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15. Isolation and purification of plant genomic DNA

EQUIPMENT AND REAGENTS:

Equipment and reagents - not supplied with the kit	
Equipment	Description
Pipettes and pipette tips	
Vortex	
Ethanol (96-100%)	
1.5 mL microcentrifuge tubes	
Microcentrifuge	
Thermomixer	shaking water bath or rocking platform capable of heating up to 65 °C
Disposable gloves	
Liquid nitrogen or grinding mill	
Polyvinylpyrrolidone (PVP)	with lignified, polyphenol-rich plant tissues
Dithiothreitol (DTT)	with rapeseeds

METHOD DESCRIPTION:

GeneJET™ Plant Genomic DNA Purification Mini Kit is designed for rapid and efficient purification of high-quality genomic DNA from wide variety of plant species and tissue types. The kit utilizes silica-based membrane technology in the form of a convenient spin column, eliminating the need for expensive resins, toxic phenol-chloroform extractions, or timeconsuming alcohol precipitation. DNA yields vary between different species and tissues depending on genome size, ploidy, cell number, and age of tissue sample. The typical yield from the optimal source, such as young wheat leaves, is 30-32 µg from 100 mg of tissue.

Samples are lysed in supplied Lysis Buffers in the presence of RNase A. Proteins and polysaccharides are removed by Precipitation Solution. The lysate is then mixed with the Plant gDNA Binding Solution, ethanol and loaded on the purification column where the DNA binds to the silica membrane. Impurities are effectively removed by washing the column with the prepared wash buffers. Genomic DNA is then eluted under low ionic strength conditions with the Elution Buffer.

The standard procedure takes less than 30 minutes following cell lysis and yields purified DNA of more than 30 kb in size.

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ASSAY PROTOCOL:

1. Pipette 350 µL of Lysis Buffer A into 1.5 mL microcentrifuge tube.
2. Weigh the plant tissue - use up to 100 mg of fresh or frozen tissue; up to 20 mg of lyophilized tissue. Place up to 100 mg of plant tissue into liquid nitrogen and grind thoroughly with a mortar and pestle.
3. Immediately transfer the tissue powder into a 1.5 mL microcentrifuge tube containing 350 µL of Lysis Buffer A. Vortex for 10-20 s to mix thoroughly.
4. Add 50 µL of Lysis Buffer B and 20 µL RNase A and vortex for 10-20 s to mix thoroughly.
5. Incubate the sample 10 min at 65 °C vortexing occasionally or use a shaking water bath, rocking platform or thermomixer.
6. Add 130 µL of Precipitation Solution and mix by inverting the tube 2-3 times. Incubate 5 min on ice or in the fridge.
7. Centrifuge for 5 min at ≥20,000 □ g (≥14,000 rpm).
8. Collect the supernatant (usually 450-550 µL) and transfer to the clean microcentrifuge tube (not provided). Add 400 µL of Plant gDNA Binding Solution and 400 µL of 96% ethanol and mix well.
9. Transfer half of the prepared mixture (600-700 µL) to the spin column. Centrifuge for 1 min at 6,000 □ g (~8,000 rpm). Discard the flow-through solution and apply the remaining mixture onto the same column. Centrifuge for 1 min at 6,000 □ g (~8,000 rpm).
10. Add 500 µL of Wash Buffer I to the column (ensure ethanol has been added to Wash Buffer I). Centrifuge for 1 min at 8,000 □ g (~10,000 rpm). Discard the flow-through and place the column back into the collection tube.
11. Add 500 µL of Wash Buffer II to the column (ensure ethanol has been added to Wash Buffer II).
12. Centrifuge for 3 min at maximum speed ≥20,000 □ g (≥14,000 rpm).
13. Discard the collection tube containing the flow-through solution and transfer the column to a sterile 1.5 mL microcentrifuge tube.
14. To elute genomic DNA, add 100 µL of Elution Buffer to the centre of the column membrane, incubate for 5 min at room temperature and centrifuge for 1 min at 8,000 □ g (~10,000 rpm).
15. Perform a second elution step using 100 µL Elution Buffer. You may perform the second elution using the same elution tube or in a different tube. The purified DNA is ready to be used in downstream applications or stored at -20 °C.

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INTERPRETATION OF THE RESULTS:

Quality and quantity of the isolated DNA is performed on a 1 % agarose gel and/or by using a BioDrop DUO UV / VIS spectrophotometer.

DEVICE PERFORMANCE:

Isolated DNA can be used directly in PCR, qPCR, Southern blotting and enzymatic reactions.

LITERATURE:

1. Vogelstein, B. and Gillespie, D. 1979. Preparative and analytical purification of DNA from agarose, Proc. Natl. Acad. Sci. USA, 76, pp. 615-619.
2. Marko, M.A., Chipperfield, R. and Birnboim, H.C. 1982. A procedure for the large-scale isolation of highly purified plasmid DNA using alkaline extraction and binding to glass powder, Anal. Biochem., 121, pp. 382-387.
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16. Kirby-Bauer Disk Diffusion Susceptibility/Resistance Testing

EQUIPMENT AND REAGENTS:

Equipment	
Equipment	Description
Biological Safety Cabinet	Best choice Biological Safety Cabinet Class II or higher (any manufacturer) with gas connection, HEPA filters, electricity.
MALDI-ToF MS Bruker Biotyper	Minimal requirements: Microflex LT from Bruker Daltonics with Biotyper software and available spectral database of microorganisms. Software requirements: Biotyper, flexControl
Pipettes	Range from 1 µl to 1000 µl
Reagent and Materials	
100% Ethanol-Sterile Solution	SIGMA or equivalent
Sterile WFI Quality Cell Culture Grade Water	Fisher Scientific or equivalent
Growing media by tested bacterial groups	Growing media is depended on cultivated bacterial strain (any manufacturer)
Plates and slants	Fisher Scientific or equivalent
Reusable polished steel MALDI target plate with 96 sample positions	Bruker Daltonics (Stainless steel polished 96-plate target)
Bruker matrix HCCA (HCCA = α-Cyano-4-hydroxycinnamic acid)	SIGMA (Cat. #C8982) or equivalent
70% Formic Acid	SIGMA (Cat. #00940) or equivalent
Acetonitrile suitable for mass spectrometry	SIGMA (Cat. #34851) or equivalent
TFA – trifluoroacetic acid suitable for HPLC ≥ 99.0%	SIGMA (Cat. #302031)or equivalent
Bacterial test standard (BTS)	Bruker Daltonics (*E. coli* DHB alpha extract) - standard
Bruker Standard Solvent	Sigma Aldrich or can be prepared by standard bruker procedure
1µl Culture loops	Any manufacturer (metal or plastic)
Gloves	Standard nitrile gloves (any manufacturer)
Sterile filter tips	1mL, 100µL, 1 µL
Microtubes	1.5ml microtubes (Ependorf tubes)

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METHOD DESCRIPTION:

The purpose of the Kirby-Bauer disk diffusion susceptibility test is to determine the sensitivity or resistance of pathogenic aerobic and facultative anaerobic bacteria to various antimicrobial compounds. The pathogenic organism is grown on Mueller-Hinton agar in the presence of various antimicrobial impregnated filter paper disks. The presence or absence of growth around the disks is an indirect measure of the ability of that compound to inhibit that organism.

ASSAY PROTOCOL:

Preparation of Mueller-Hinton plate

- Allow a MH agar plate (one for each organism to be tested) to come to room temperature. It is preferable to allow the plates to remain in the plastic sleeve while they warm to minimize condensation.
- If the surface of the agar has visible liquid present, set the plate inverted, ajar on its lid to allow the excess liquid to drain from the agar surface and evaporate. Plates may be placed in a 35°C incubator or in a laminar flow hood at room temperature until dry (usually 10 to 30 minutes).
- Appropriately label each MH agar plate for each organism to be tested.

Preparation of inoculum

- Using a sterile inoculating loop or needle, touch four or five isolated colonies of the organism to be tested.
- Suspend the organism in 2 ml of sterile saline.
- Vortex the saline tube to create a smooth suspension.
- Adjust the turbidity of this suspension to a 0.5 McFarland standard by adding more organism if the suspension is too light or diluting with sterile saline if the suspension is too heavy.
- Use this suspension within 15 minutes of preparation.

INTERPRETATION OF THE RESULTS:

- Using the published EUCAST guidelines, determine the susceptibility or resistance of the organism to each drug tested (link: www.eucast.org). Note that there are different charts for different organisms.
- For each drug, indicate on the recording sheet whether the zone size is susceptible (S), intermediate (I), or resistant (R) based on the interpretation chart.
- The results of the Kirby-Bauer disk diffusion susceptibility test are reported only as susceptible, intermediate, or resistant. Zone sizes are not reported to physicians.

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17. Microsatellite analysis of plant genomic DNA

EQUIPMENT AND REAGENTS:

Equipment	
Equipment	Description
Thermocycler	Biometra
Vertical electrophoretic unit	Hoefer
Pipettes	Range from 1 µl to 1000 µl
Reagent and Materials	
GoTaq® G2 Green Master Mix	Fisher Scientific
Primer F	
Primer R	
Acetic acid	SIGMA
Acrylamide	SIGMA
Urea	SIGMA
Bisacrylamide	SIGMA
Ammoium persulphate	SIGMA
TEMED	SIGMA
EDTA	SIGMA
Tris-HCl	SIGMA
Formaldehyd	SIGMA
Silver nitrate	SIGMA
Sodium tiosulfate	SIGMA
Sodium carbonate	SIGMA
boric acid	SIGMA
Gloves	
Sterile filter tips	1mL, 100µL, 200µL, 20µL, 10 µL
Microtubes	0.5ml microtubes

METHOD DESCRIPTION:

Microsatellite analysis are based on PCR reaction, uses locus specific primers flanking microsatellites and they detect length polymorphism (variability) caused by difference in the number (length) of tandem repeats in study locus between genotypes.

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ASSAY PROTOCOL:

Polymerase Chain Reaction (PCR) and Gel Electrophoresis

Microsatellite analyzes are perfomed with genome and locus specific 17-22 oligonucleotide sequences (for wheat according to Devos et., 1995, Röder et al., 1998). The amplification conditions are set for individual microsatellite markers. PCR reactions are performed in a thermocycler.

The PCR amplicons (5µl) are resolved by electrophoresis on 6.0 % denaturating polyacrylamide gel and run with 1.0 x TBE buffer. Electric voltage and time were different for each marker. The electric voltage was set to 2000 V and time of electrophoretic separation differs for each marker (3-4 hours) influenced by the predicted size of fragments. After electrophoresis, gels are fixed and stained with silver nitrate (Bassam et al., 1991). Final PCR amplicons are scanned in UVP PhotoDoc-t® camera system. The size of alleles is determined by comparing with 10 bp standard lenght marker (Invitrogen: 100-330 bp). Each band is treated as a single allele.

INTERPRETATION OF THE RESULTS:

Each reproducible band was visually scored for the presence (1) or absence (0) for all genotypes. For determination of the genetic relationships between rye genotypes a dendrogram was used. The dendrogram was constructed based on principle of hierarchical cluster analysis using UPGMA (Unweighted Pair Group Method using arithmetic Averages) algorithm on the basis of Jaccard's coefficient in statistical program.

Frequencies of incidence of all polymorphic alleles were calculated and used for determination of statistical parameters: diversity index (DI) (Weir, 1990), probability of identity (PI) (Paetkau et al., 1995) and polymorphic information content (PIC) (Weber, 1990).

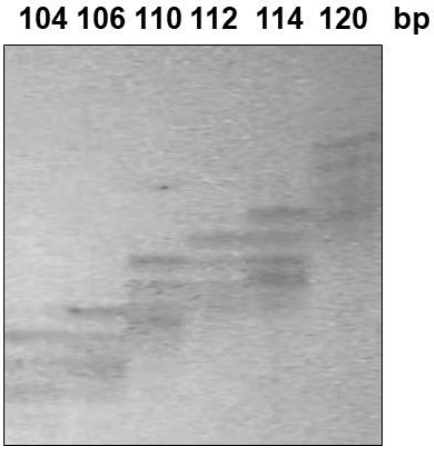

Fig.1 Microsatellite alleles in the PAGE gel stained with silver nitrate

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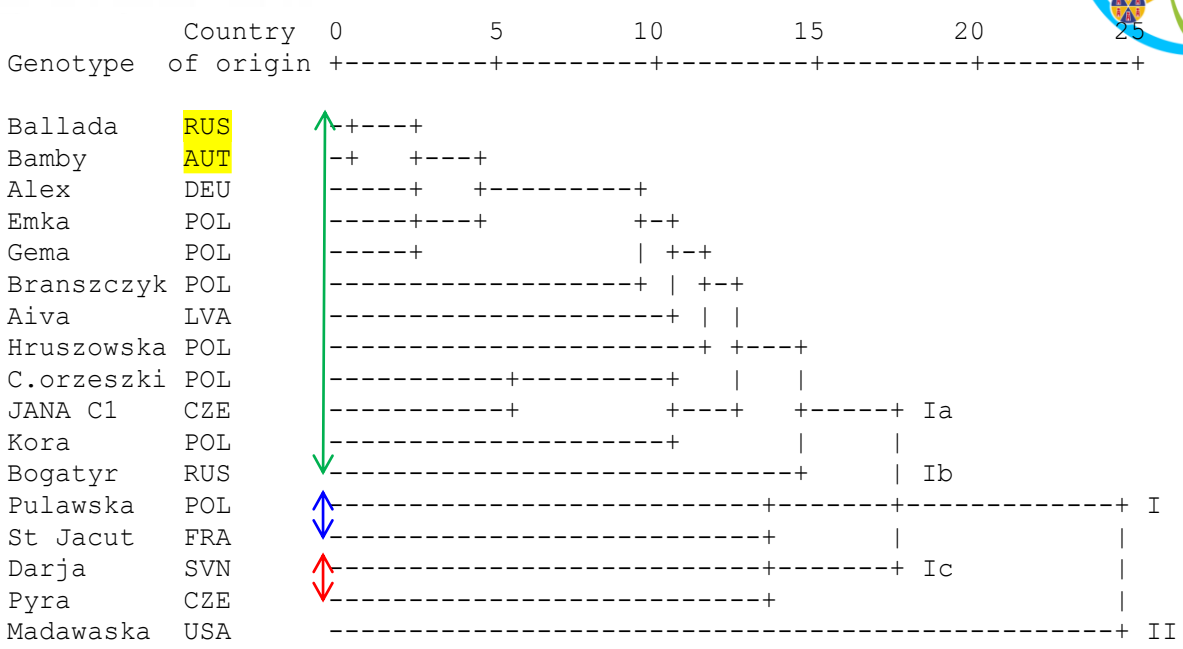

Fig.2 Dendrogram constructed based on principle of hierarchical cluster analysis using UPGMA algorithm using microsatellite primer pairs

LITERATURE:

1. Bassam, B.J., Caetano-Anolles, G., Gresshoff, P.M. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Analytical Biochemistry *196*: 80-83.
2. Devos, K.M., Bryan, G.J., Collins, A.J. 1995. Application of two microsatellite sequences in wheat storage proteins as molecular markers. Theoretical and Applied Genetics *90*: 247-252.
3. Paetkau, D., Calvert, W., Stirling, I., Strobeck, C. 1995. Microsatellite analysis of population structure in Canadian polar bears. Molecular Ecology. *4*: 347-354.
4. Röder, M.S., Korzun, V., Wendehake, K., Plaschke, J., Tixier, M.H., Leroy, P., Ganal, M.V. 1998. A microsatellite map of wheat. Genetics. *149*: 2007-2023.
5. Weber, J.L. 1990. Informativeness of human $(dC\text{-}dA)_n$ x $(dG\text{-}dT)_n$ polymorphism. Genomics. *7*: 524-530.
6. Weir, B.S. 1990. Genetic data analysis. Sinauer Associated, Sunderland, Mass.

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18. MTT assay protocol for cell viability and proliferation

EQUIPMENT AND REAGENTS:

Equipment and reagents - not supplied with the kit	
Equipment	Description
96-well Elisa Plate Reader	cat. no 51119000, Multiscan
CO2 incubator	Adjustable to 37 °C
Digital Mini Rotator	cat. no 88882007, Thermo Scientific
Micro pipettes	Range from 100 µL to 300 µL
Optional equipment	
Equipment	Description
Multichannel Pipette	Range from 100 µL to 300 µL
Reagent and Materials	
Samples	
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	
Dimethyl sulfoxide	
Nutrient Mixture F-12 Ham	
96 well plate	
Disposable gloves	
Sterile filter tips	
15 and 50 ml tubes	
Multichannel Pipette Reservoir	

METHOD DESCRIPTION:

The MTT assay is used to measure cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. This colorimetric assay is based on the reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) to purple formazan crystals by metabolically active cells (Figure 1).6,7,35 The viable cells contain NAD(P)H-dependent oxidoreductase enzymes which reduce the MTT to formazan. The insoluble formazan crystals are dissolved using a solubilization solution and the resulting-colored solution is quantified by measuring absorbance at 500-600 nanometers using a multi-well spectrophotometer. The darker the solution, the greater the number of viable, metabolically active cells. This non-radioactive, colorimetric assay system using MTT was first described by Mosmann and improved in subsequent years by several other investigators. MTT can be used for multiple applications, such as:

- Quantification of cell growth and viability.

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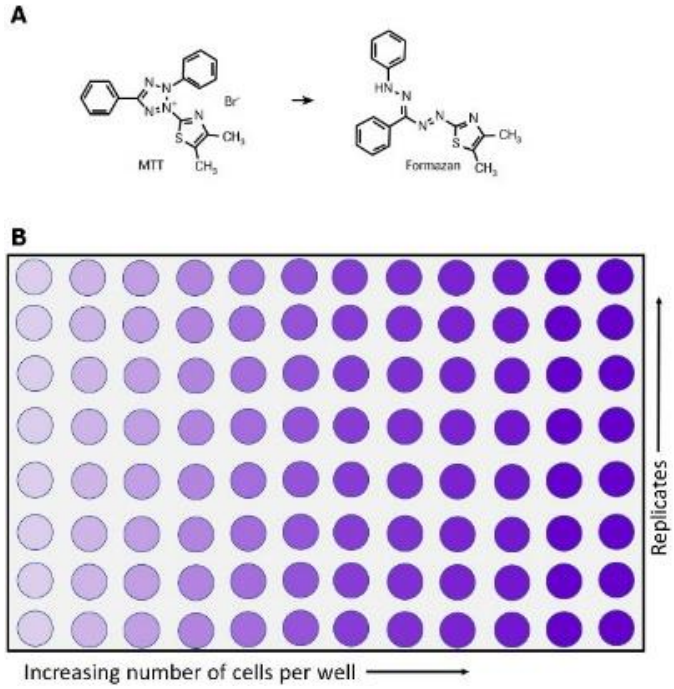
- Measurement of cell proliferation in response to growth factors, cytokines and nutrients.
- Measurement of cytotoxicity. Examples are the quantification of tumor necrosis factor-a or -b or macrophage induced cell death and the assessment of cytotoxic or growth inhibiting agents such as inhibitory antibodies.
- To study cell activation.



Metabolism of MTT to a formazan salt by viable cells as shown in a chemical reaction (A) and in a 96-well plate (B).

ASSAY PROTOCOL:

1. Discard media from cell cultures. For adherent cells, carefully aspirate the media. For suspension cells, spin the 96 well plate at 1,000 x g, 4°C for 5 minutes in a microplate-compatible centrifuge and carefully aspirate the media.
2. Add 50 µL of serum-free media and 50 µL of MTT solution into each well.
3. Incubate the plate at 37°C for 3 hours.
4. After incubation, add 150 µL of MTT solvent (DMSO) into each well.
5. Wrap plate in foil and shake on an orbital shaker for 15 minutes. Occasionally, pipetting of the liquid may be required to fully dissolve the MTT formazan.
6. Read absorbance at OD=590 nm. Read plate within 1 hour.

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DATA ANALYSIS:

Cell proliferation assays

1. Average the duplicate reading for each sample.
2. Subtract the culture medium background from your assay reading. This is the corrected absorbance.
3. The amount of absorbance is proportional to cell number.

For cell counting, a standard curve can be established with known cell number and fixed incubation times with the assay reagent.

Cell cytotoxicity assays

1. Average the duplicate reading for each sample.
2. Subtract the culture medium background from your assay readings. This is the corrected absorbance.
3. Calculate percentage cytotoxicity with the following equation, using corrected absorbance: % cytoxicity = (100 x (control - sample))

LITERATURE:

1) Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays; Journal of Immunological Methods, Vol. 65, No. 1-2, pp. 55-63.
2) Abcam. 2021. MTT assay protocol. https://www.abcam.com/kits/mtt-assay-protocol

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19. NBT assay protocol

EQUIPMENT AND REAGENTS:

Equipment and reagents - not supplied with the kit	
Equipment	Description
96-well Elisa Plate Reader	cat. no 51119000, Multiscan
CO2 incubator	Adjustable to 37 °C
Digital Mini Rotator	cat. no 88882007, Thermo Scientific
Micro pipettes	Range from 100 µL to 300 µL
Optional equipment	
Equipment	Description
Multichannel Pipette	Range from 100 µL to 300 µL
Reagent and Materials	
Samples	
Nitro blue Tetrazolium Chloride	
KOH	
Nutrient Mixture F-12 Ham	
96 well plate	
Disposable gloves	
Sterile filter tips	
15 and 50 mL tubes	
Multichannel Pipette Reservoir	

METHOD DESCRIPTION:

Nitro blue tetrazolium chloride (NBT) can be used to assess cell viability as a function of redox potential. Nitroblue tetrazolium (NBT) undergoes reduction by O2•− to form diformazan, a dark blue insoluble precipitate.31 Similar to DHE, NBT detects intracellular O2•−; however, it is less sensitive and specific for O2•− than is DHE. NBT is susceptible to reduction by several tissue reductases,18 and therefore specificity for O2•− should be confirmed by inhibition of NBT staining by SOD. Like lucigenin, NBT also has been shown to artifactually generate O2•− by autoxidation.32 For these reasons, detection of O2•− in biologic samples should not rely exclusively on NBT reduction. Tissue samples are placed in phenol red-free medium or HBSS with or without addition of inhibitors (SOD mimetic, DPI, etc.) at 37 °C for 30 min. NBT (0.1 mM) is added and the incubation is continued for 1–3 hr, depending on the amount of O2•− production. An equal volume of 0.5 N HCl is added to stop the reaction and the tissues are rinsed with cold PBS.

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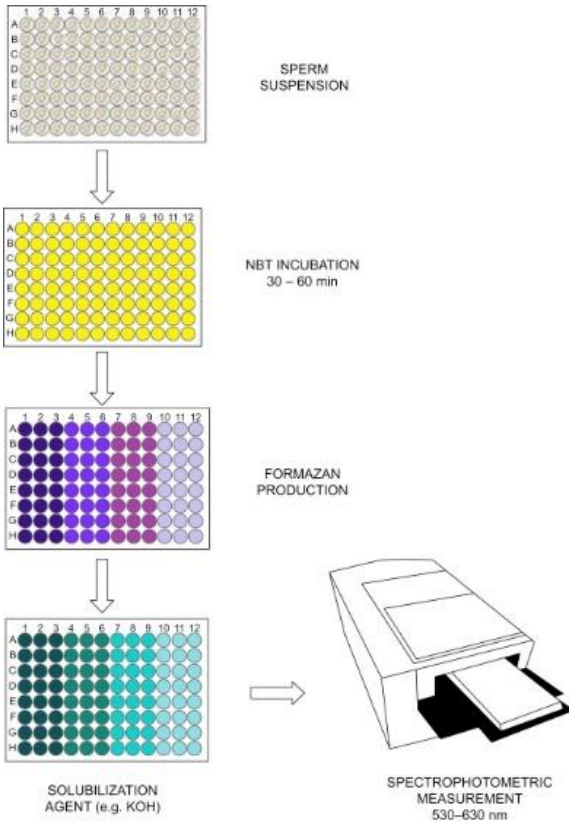

Metabolism of NBT and procedure of NBT assay

ASSAY PROTOCOL:

1. Discard media from cell cultures. For adherent cells, carefully aspirate the media. For suspension cells, spin the 96 well plate at 1,000 x g, 4°C for 5 minutes in a microplate-compatible centrifuge and carefully aspirate the media.

   An alternative method is to add an equal volume of NBT solution to the existing media in the culture. Ensure that the same volume of existing media is present for each sample.

2. Add 50 µL of serum-free media and 50 µL of NBT solution into each well.
3. Incubate the plate at 37°C for 3 hours.
4. After incubation, add 150 µL of solubilization agent (KOH) into each well.
5. Wrap plate in foil and shake on an orbital shaker for 60 minutes.
6. Read absorbance at OD=590 nm. Read plate within 2 hours.

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DATA ANALYSIS:

Cell proliferation assays

1. Average the duplicate reading for each sample.
2. Subtract the culture medium background from your assay reading. This is the corrected absorbance.

LITERATURE:

1. Tvrdá, E. 2019. NBT Test. In: Oxidants, Antioxidants and Impact of the Oxidative Status in Male Reproduction. Editor(s): Ralf Henkel, Luna Samanta, Ashok Agarwal. Academic Press, 2019, pp. 195-205.
2. Goldbio. 2018. NBT Assay: Brice Weinberg Protocol. https://www.goldbio.com/uploads/documents/472cc2c28235b27618cf97ce8ead05c3.pdf

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20. PCR based techniques to study plant genetic diversity using different molecular markers

EQUIPMENT AND REAGENTS:

Equipment and reagents	
Equipment	Description
Thermal cycler	
Horizontal electrophoresis unit	
UVP PhotoDoc-t® camera system	
Micro pipettes	Range from 2 µl to 1000 µl
PCR chemicals	Taq Polymerase, Buffer, dNTPs, primers
Deionised water	
Disposable gloves	
Sterile filter tips	
250ml bottle	
PCR microtubes	200 µl or 500 µl depend on thermocycler

METHOD DESCRIPTION:

The analysis consists of two main procedure steps.

- PCR amplification of DNA fragments with primers (RAPD, SCoT and others)
- Horizonatl gel electrophoresis of PCR fragments and detection

PCR

PCR reaction contain isolated plant DNA, Taq polymerase, dNTPs, deionised water and primer. One primer is added to the PCR mix. Amplification products of 300 – 3000 bp can be expected. To evaluate genetic diversity of chosen plant species more SCoT primers must be used.

HORIZONATL GEL ELECTROPHORESIS OF PCR FRAGMENTS AND DETECTION

Amplified fragments are separated in 1.5 % agarose gels in 1× TBE buffer. The gels are stained with ethidium bromide and documented using gel documentation system UVP PhotoDoc-t®. Size of amplified fragments is determined by comparing with standard length marker Quick-Load® Purple 2-Log DNA ladder.

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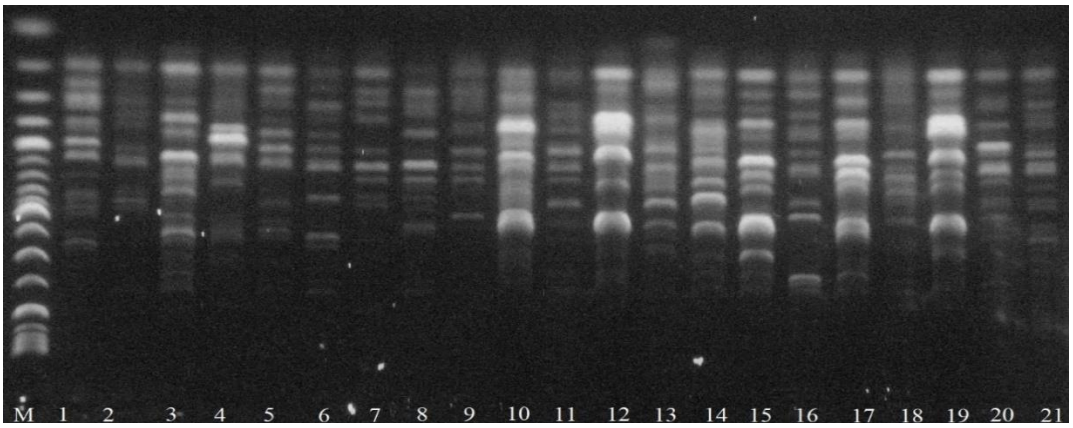

Fig.1 Electrophoreogram of SCoT marker of *Fagopyrum esculentum* genotypes.
Note: M is Quick-Load® Purple 2-Log DNA ladder. 1 – 21 are genotypes of *Fagopyrum esculentum*

INTERPRETATION OF THE RESULTS:

The amplified bands are evaluated as present (one) or absent (zero) in all genotypes for each visible fragment and from the data obtained binary matrix is prepared. A dendrogram based on hierarchical cluster analysis using the unweighted pair group method with arithmetic average (UPGMA) with the statistical software is constructed.

DEVICE PERFORMANCE

All evaluation experiments with respect to the assay specificity and sensitivity are performed with plant DNA extracted from seedlings of the respective plant species. Molecular markers are evaluated if they are informative and effective. Molecular markers can be used to identify and characterize plant genotypes and also to study genetic diversity among the plant genotypes.

LITERATURE:

1. Collard, B. C. Y., Mackill, D. J. 2009. Start codon targeted (SCoT) polymorphism: a simple, novel DNA marker technique for generating gene-targeted markers in plants. *Plant Mol. Biol. Rep.*, vol. 27, pp. 86-93
2. Gajera, H. P., Bambharolia, R. P., Domadiya, R. K., Patel, S. V., Golakiya, B. A. 2014. Molecular characterization and genetic variability studies associated with fruit quality of indigenous mango (*Mangifera indica* L.) cultivars. *Plant Syst Evol*, vol. 300, pp. 1011-1020.

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21. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

EQUIPMENT AND REAGENTS:

Equipment	
Equipment	Description
Vertical electrophoretic unit	Hoefer SE 600 Chroma
Scaner Bio-Rad GS-800 Calibrated Densitometer	
Pipettes	Range from 1 µl to 1000 µl
Reagent and Materials	
Tris-HCl	SIGMA
Mercaptoethanol	SIGMA
Acrylamide	SIGMA
Bisacrylamide	SIGMA
Ethanol	SIGMA
SDS	SERVA
Ammonium persulphatum	SIGMA
Coomasie briliant blue R250	SIGMA
Trichloroacetic acid	SIGMA
Acetic acisd	SIGMA
Glycerol	SIGMA
Glycine	SIGMA
TEMED	SIGMA
Pyronin G	SIGMA
Spectra Multicolor Broad Range Protein Ladder	Thermo Scientific
Gloves	
Sterile filter tips	1mL, 100µL, 1 µL
Microtubes	1.5ml microtubes

METHOD DESCRIPTION:

Polyacrylamide gel electrophoresis in the presence of SDS is universal and the most widely used technique for the analysis and separation of proteins and RNA molecules. The polyacrylamide gel consists of two parts with different pore sizes, starting and a separating gel. Sodium dodecyl sulfate, which forms, is added to the polyacrylamide gel complex with the proteins, thus balancing their charges and the protein molecules separating only on the basis of their molecular weight. SDS-PAGE is a relatively inexpensive method, however

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quantification of isolated protein fractions is a problematic and separated protein should be visualized by silver staining, Coomassie Brilliant Blue or fluorescent dyes (Hulín et al., 2008).

ASSAY PROTOCOL:

Glutenin extraction

Glutenin extraction solutions

Storage solution:

1M Tris-HCl pH 6,8	12,5 ml
Glycerol	20 ml
H_2O	24,1 ml
Pyronin G	20 mg

Extraction solution:

Storage solution	4,25 ml
2-mercapthoethanol	0,75 ml
H_2O	10 ml

Glutenin extraction procedure

50 mg of homogenized grain x 8 µl extraction solution
30 min extraction 100 °C 1200 rpm by horizontal shaker
10 min centrifugation 15 000 rpm 4 °C
Supernatant to new tube

SDS glutenin separation

Solutions

1M Tris-HCl ph 6,8	12,114 g Tris up to 100 ml H_2O, HCl pH ekvilibration
1M Tris-HCl ph 8,8	12,114 g Tris up to 100 ml H_2O, HCl pH ekvilibration
Starting AA-BIS	7,29 g acrylamide, 0,125 g bisacrylamide to 100 ml H_2O
Separating AA-BIS	54,49 g acrylamide, 0,72 g bisacrylamide to 250 ml H_2O
10 % SDS	5 g sodium dodecyl suphate up to 50 ml H_2O
2 % APS	0,02 g ammonium persulphatum, 1 ml H_2O
4 % APS	0,04 g ammonium persulphatum, 1 ml H_2O
TEMED	
Electrophoretic buffer	28,2 ml glycine, 6 g Tris, 2 g SDS up to 2 L H_2O

Gel preparation

Starting gel:

Tris-HCl pH 6,8	1,236 ml
Starting AA-BIS	8,3 ml
10% SDS	0,1 ml
2 % APS	0,8 ml
TEMED	0,03 ml

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Separating gel:

Tris-HCl pH 8,8	11,43 ml
AA-BIS Separating	17,48 ml
10 % SDS	0,3 ml
4 % APS	0,76 ml
TEMED	0,06 ml

Electrophoretic separation procedure

1 hour	5 mA	500 V	50 W
19 hour	10 mA	500 V	50 W

Gel staining

Solutions:

0,5 % Coomasie briliant blue R250	0,5 g CBB R250 up to 100 ml ethanol
10 % trichloroacetic acid	200 g trichloroacetic acid up to 100 ml H_2O

Staining solution:

190 ml	10 % trichloroacetic acid
10 ml	0,5 % Coomasie briliant blue R250

Staining procedure:

Gel immerse for 24 h in laboratory temperature in staining solution

Gel destaining

Destaining solution:

Acetic acid	200 ml
Glycerol	40 ml
H_2O	up to 2 L

Destaining procedure:

Gel immerse for 24 h in laboratory temperature in destaining solution

INTERPRETATION OF THE RESULTS:

Interpretation of results is based on comparison of the separated molecules in gel according to standard sample and according to marker of molecular weight by software. The grain storage proteins of the analyzed plant genotypes separated in SDS-PAGE based on their molecular weight to the high molecular weight glutenin subunits (HMW-GS) at the top polyacrylamide gel, low molecular weight glutenin subunits (LMW-GS) in the middle of the gel and residual albumins and globulins on the bottom of the gel. Separated protein molecules is possible to compare within two or more samples which obtain differentiation of samples. There is also possibility to investigated changes in proteome of individual sample in different conditions.

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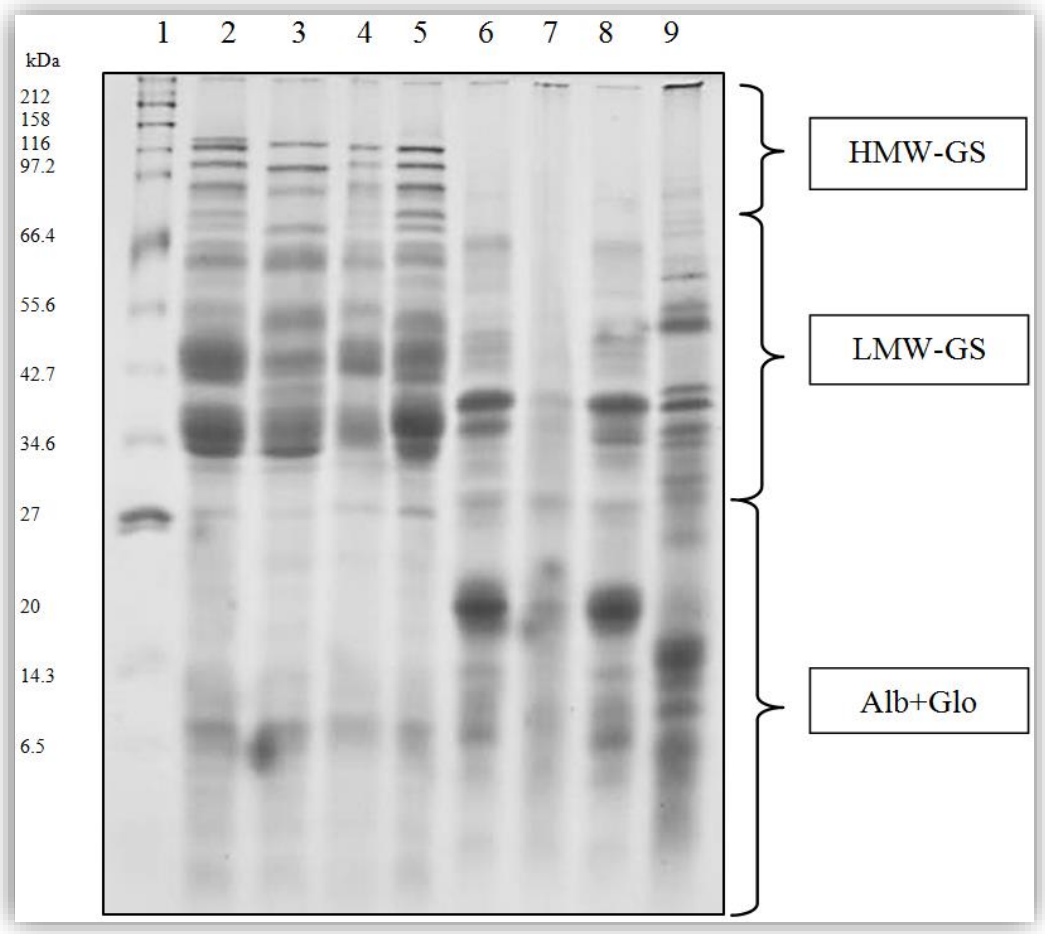

Fig 1. SDS-PAGE electrophoreogram

LITERATURE:

1. Hulín, P., Dostálek, P., Hochel, I. 2008. Metody stanovení lepkových bílkovin v potravinách. In Chemické listy [online], vol. 102, no. 5, pp. 327-337.
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22. Sperm chromatin dispersion test

EQUIPMENT AND REAGENTS:

Equipment and reagents - not supplied with the kit	
Equipment	Description
Fluorescent microscope	x 60 magnification
Optical microscope	x 60 magnification
Fridge	
Thermal block or water bath	Adjustable to 37°C and 95 °C
Micropipettes	Range from 1 µL to 100 µL
Haemocytometer	
Fume hood	
Reagent and Materials	
Halomax kit	HT-XX, HaloTech DNA
Petri dishes or similar tray	
Pipette tips	
Disposable gloves	
Semen extender	Depending on the species
Fluorescent dye for fluorescent microscopy	SybrGreen, ethidium bromide or alternatives for nucleic stain (DAPI is not suitable for this technique)
Anti-fading medium	Vectashield or alternatives
Ethanol	70% and 100%
Distilled water	
Brighfield staining kit for optical microscopy	HaloTech DNA
Glass coverslips	24x24 mm

METHOD DESCRIPTION:

The protocol is based on the differential response of sperm chromatin with or without fragmented DNA to a protein depletion treatment. In the absence of a massive DNA breakage, the removal of nuclear proteins produces intensely stained nucleoids with very small haloes of DNA loops emerging from a central and compact core. However, nucleoids from sperm containing fragmented DNA show a big and faintly stained halo of diffusion of DNA fragments emerging from a residual central core.

DOI: https://doi.org/10.15414/2023.9788055226248

ARRAY DESCRIPTION:

1. Set the Lysis Solution (LS) at room temperature (22°C)

2. Dilute the sperm sample in an appropriate extender to a concentration of 15-20 million sperm/mL

3. Place an Eppendorf tube containing agarose (500 µL) in the float and incubate at 95-100°C for 5 minutes or until the agarose is fully melted.

4. Transfer the agarose Eppendorf tube, with the float, in a water bath at 37°C and leave it for 5 minutes until the temperature has equilibrated.

5. Meanwhile, aliquot 25 µL of each sperm sample in an empty Eppendorf tube.

6. Transfer 50 µL of liquefied agarose into the tube with the semen sample and mix gently. Maintain the tubes at 37°C.

7. Place a drop of 1.5-2 µL of the cell suspension onto the marked wells and cover 4 wells with a 24x24 mm glass coverslip. Press gently, avoiding air bubble formation.

8. Slides must be held in a horizontal position throughout the entire process.

9. Place the slide on a cold surface and transfer into the fridge at 4°C for 5 minutes to solidify the agarose.

10. Take the slide out of the fridge and remove the coverslip by sliding it off gently. All subsequent processing must be performed at room temperature (22°C).

11. Place the slide horizontally into a Petri dish or similar tray.

12. Apply Lysis Solution over on the wells making sure that they are fully immersed. Incubate for 5 minutes. Drain by tilting and place the slide horizontally on the top of the float.

13. Wash covering the slide for 5 minutes with abundant distilled water. Drain by tilting and place the slide horizontally on the top of the float.

14. Dehydrate the slides with 70% ethanol and incubate for 2 minutes. Dry and apply 100% ethanol for 2 minutes. Drain and allow to dry.

15. After drying, the slides may be kept at room temperature in a dry and dark place for several months.

16. For the visualization and analysis under the fluorescent microscope, the slides are stained with appropriate fluorochormes. For example, SybrGreen dyes prepared at a final concentration of x40 and mixed 1:1 in an antifading mounting medium are recommended. A final mixed volume of 2 µL is enough to stain one well.

17. For optical microscopy, the Brightfield Staining Kit is recommended. For the staining procedure, the slide is incubated in the staining solution A for 6 minutes, followed by incubation in the staining solution B for 7 minutes. The slides are then left to dry in vertical position and checked under brightfield microscopy.

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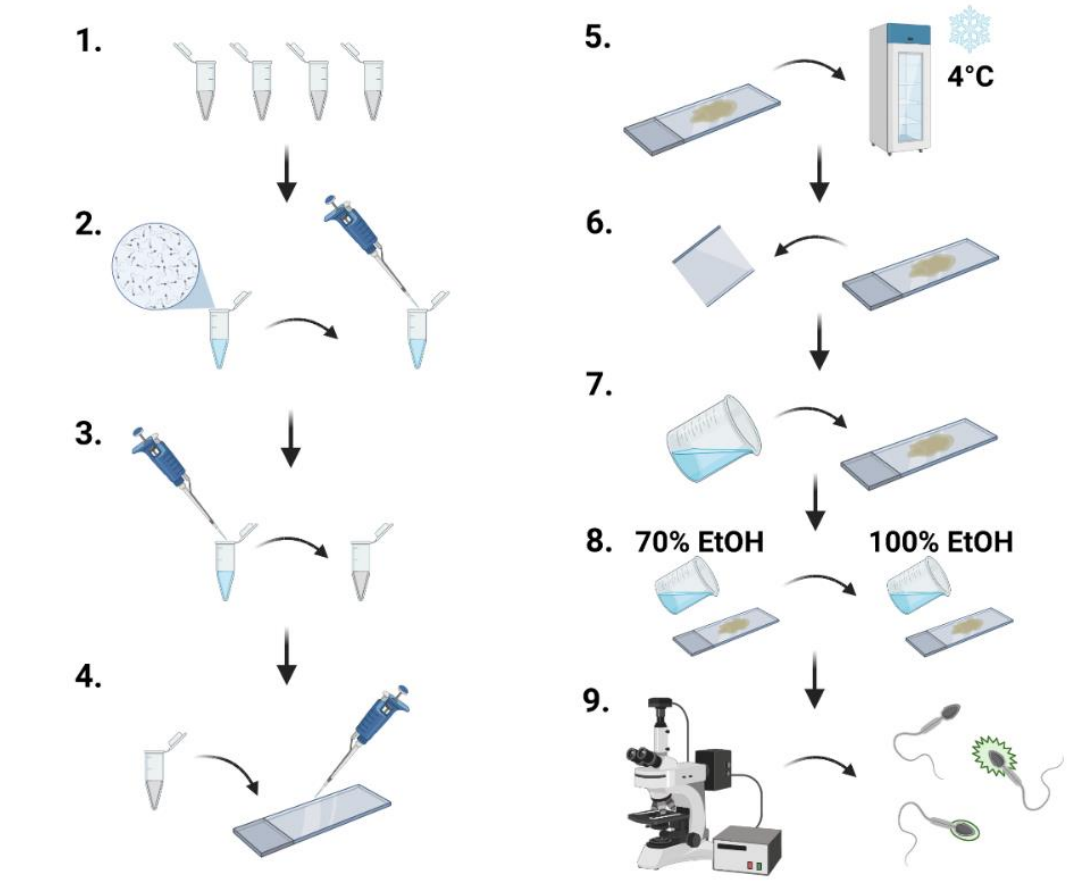

A simple summary of the protocol

INTERPRETATION OF THE RESULTS:

Sperm classification

Following protein depletion, sperm nuclei appear as nucleoids with a central core surrounded by a peripheral halo of chromatin/DNA. The analysis of a minimum number of 300 sperm cells per sample is recommended. The criteria for classification are as follows:

Sperm with fragmented DNA – sperm showing nucleoids with a large and spotty halo of chromatin dispersion.

Sperm without fragmented DNA – sperm showing nucleoids with a small and compact halo of chromatin dispersion.

Index of fragmentation = (sperm with fragmented DNA ÷ total number of spermatozoa) x 100

DOI: https://doi.org/10.15414/2023.9788055226248

LITERATURE:

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2. Gosalvez, J., Fernandez, J.L., Gosalbez, A., Arroyo, F., Agarwal, A. and Lopez-Fernandez, C. 2007. Dynamics of sperm DNA fragmentation in mammalian species as assessed by the SCD methodology; Fertility and sterility, Vol. 88, Suppl. 1, pp. S365.

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23. Sperm viability assessment by flow cytometry

EQUIPMENT AND REAGENTS:

Equipment and reagents - not supplied with the kit	
Equipment	Description
FACS Calibur; BD Biosciences	
Centrifuge	Micro 200 R
Pipettes	Range from 2 µl to 1000 µl
Optional equipment	
Equipment	Description
Vortex	Vortex V1 plus, Biosan
Test tube rack holder	Test Tube Rack Holder 28 Sockets 10/15/50ml Centrifuge Tube Rack Laboratory Plast
Reagent and Materials	
Reagents for sample staining	
Phosphate-buffered saline (PBS)	
Yo-Pro-1	
Propidium iodide	
Gloves	
Sterile filter tips	
Falcon tubes	5ml test tubes
Microtubes	1.5ml microtubes
Reagents for FACS	
Sheet fluid for FACS	
Reagent for FACS cleaning	

METHOD DESCRIPTION:

Viability of sperm was determined using Yo-Pro-1 dye and propidium iodide (PI). The semen samples were washed and centrifuged in PBS (Life Technologies, Slovak Republic) at 600 *g* for 5 min and the supernatant was discarded. Yo-Pro-1 solution (Molecular Probes, Lucerne, Switzerland) at concentrations of 100 µmol/l and 50 µg/ml PI solution (Molecular Probes, Lucerne, Switzerland) were used for detection of apoptosis. Samples were vortexed and incubated in the dark at room temperature for 15 min. Then, samples were washed in PBS and centrifuged at 600 *g* for 5 min. The supernatant was discarded and flow cytometry was performed. The stained semen samples were analysed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA). At least 10,000 events (spermatozoa) were analysed in each sample. The emitted green fluorescence of Yo-Pro-1 positive cells and red fluorescence of PI-positive cells were recorded in the FL-1 and FL-3 channels, respectively.

DOI: https://doi.org/10.15414/2023.9788055226248

ARRAY DESCRIPTION:

Fluorescence immunophenotyping is a common technique for characterizing different cell types or populations. This method uses fluorescent dyes to identify and characterize different physiological cell processes such as viability, acrosomal status, DNA changes, ROS, etc. Flow cytometry is an optical laser technology that analyzes the physical and fluorescent properties of sperm in suspensions in real time as they flow through the instrument. This approach has several advantages over other techniques used to characterize populations in a single cell suspension, namely that it can objectively analyze millions of sperm and record data on the presence of different phenotypic changes within populations in seconds.

INTERPRETATION OF THE RESULTS:

Data analysis is a very critical part of any experiment that utilizes flow cytometry. The beginning user will probably have assistance from a dedicated flow cytometer operator when acquiring data; however, analysis of the acquired data is usually very specific to the experimental objectives. Therefore, the user is much more aware of what data will be required to achieve the experimental outcome. In order to conduct data analysis, the user must have a good working knowledge of what data analysis options are available, how to display data, and how to interpret data. List mode data are analyzed using a computer and software. The software is usually specific to flow cytometric data and is often times part of the same computer system that is used to control the instrument during acquisition. Third-party companies also offer software for data analysis. These programs provide many ways to examine data; however, there are some very useful standard ways of presenting data that are common to all types of software. These are described below.

1. Histograms are excellent tools for data analysis, because they allow the user to see the distribution of a single measured parameter for the acquired events. A histogram format is commonly used to display results from samples that were treated using a variety or panel of antibodies conjugated to the same fluorochrome.
2. It is also possible to display two parameters simultaneously, such as FSC vs SSC or FL1 vs FL2. Any combination of acquired parameters can be used to depict a two-parameter data plot. For two-parameter plots, data from a population of individual particles can be displayed in the form of dots or as contours. Dot plots display data from each particle as a dot within both coordinate axes; one dot represents one acquired event. The positions of the dots reflect the relative intensities of the two measured parameters for that event. Contour density plots display the data from a population of cells as a series of concentric lines that correlate to different cell or particle densities within the axes. Contour plots are similar to topographical maps. The power of these two various types of data displays is that they allow an investigator to visualize two measured parameters on a single plot. Dot plots

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are probably the most common type of two-parameter plots, and they are also the easiest to understand. Contour displays require more experience to interpret.

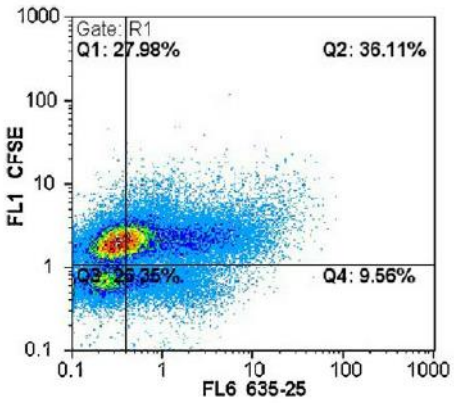

Figure 1 Representative flow cytometry dot plots for control and experimental groups. Q3 represents viable (Yo-Pro-1^{-} and PI^{-}), Q4 - apoptotic (Yo-Pro-1^{+} and PI^{-}), Q2 - dying (PI^{+}, Yo-Pro-1^{-}) and Q1 – dead (PI^{+}) spermatozoa.

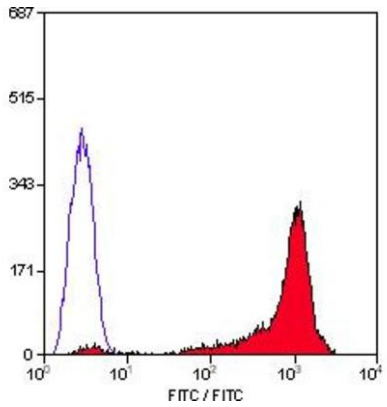

Figure 2 Representative flow cytometry histogram for control and experimental group. Fluorescent shift was caused by positivity on Yo-Pro-1 or PI.

LITERATURE:

1. Vašíček, J., Svoradová, A., Baláži, A., Jurčík, R., Macháč, M., & Chrenek, P. 2021. Ram semen quality can be assessed by flow cytometry several hours after post-fixation. Zygote, 29(2), pp. 130-137. doi:10.1017/S0967199420000581
2. Marchisio, M., Simeone, P., Bologna, G., Ercolino, E., Pierdomenico, L., Pieragostino, D., & Lanuti, P. 2021. Flow cytometry analysis of circulating extracellular vesicle subtypes from fresh peripheral blood samples. International Journal of Molecular Sciences, 22(1), p. 48.
3. Llavanera, M., Ribas-Maynou, J., Delgado-Bermúdez, A., Recuero, S., Muiño, R., Hidalgo, C. O., & Yeste, M. 2021. Sperm chromatin condensation as an in vivo fertility

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biomarker in bulls: a flow cytometry approach. Journal of Animal Science and Biotechnology, 12(1), pp. 1-12

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24. Standard and additional serum chemistry analysis

EQUIPMENT AND REAGENTS:

Equipment and reagents - not supplied with the kit	
Equipment	Description
The RX Monaco fully automated analyzer (Randox Laboratories Ltd.; Crumlin, United Kingdom) comes supplied as part of a package, with all essential components provided. These components are approved for use with the RX Monaco and make it easier for the user to conduct testing. 	
Micro pipettes	Range from 5 µL to 10 000 µL
Reagent and Materials	
Calibrations and controls material	Calibration Sera Level 3; CRP Full Range 6-Level Series; HDL/LDL Calibrator; Human Assayed Control Level 2; Human Assayed Control Level 3; CRP High Sensitivity Contro; etc. from Randox Laboratories Ltd.; Crumlin, United Kingdom
Reagents material	Albumin; ALT; ALP; ASt; Bilirubin; Ca, P, Mg, CRP, Creatinine; Ferritin; Glucose; GGT; HDL, LDL, Transferrin; Total Protein; Triglycerides; Cholesterol; Uric Acid; Urea; HbA1c; TAC, GPx, SOD, BHB; etc. from Randox Laboratories Ltd.; Crumlin, United Kingdom
Standard sample cups	2ml
Samples	Blood serum Blood plasma Whole blood (EDTA or heparin)

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METHOD DESCRIPTION:

The RX Monaco is a comprehensive testing solution that includes a wide range of tests for routine chemistries, specific proteins, lipids, antioxidants, diabetes, and veterinary testing, as well as emergency and STAT sampling. With its efficient and user-friendly platform, the RX Monaco is ideal for clinical chemistry testing in low to mid-volume settings, allowing routine and specialized testing to be consolidated onto one system. This versatile system can perform up to 170 photometric tests per hour, providing customers with convenience, performance, and confidence. Additionally, the RX Monaco offers the choice between a benchtop or floor standing system, allowing customers to select the best option to meet their unique needs.

LITERATURE:

1. https://www.randox.com/rx-monaco-overview/ (© 2023 Randox Laboratories Ltd.)

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25. Thiobarbituric acid reactive substance (TBARS) assay

EQUIPMENT AND REAGENTS:

Equipment and reagents - not supplied with the kit	
Equipment	Description
96-well Elisa Plate Reader	Cat. no 51119000, Multiscan
Thermal block or water bath	Adjustable to 100 °C
Centrifuge	With cooling
Vortex mixer	Cat. No BS-VOR-P, Benchmark Scientific
pH meter	
Micro pipettes	Range from 5 µL to 4 mL
Graduated cylinders	Range from 10 mL to 100 mL
Reagent and Materials	
2-Thiobarbituric acid	Cat. No. T5500, Sigma Aldrich
Acetic acid	20%, Cat. No. A6283, Sigma Aldrich
Sodium Hydroxide	1 mol/L, Cat. No. 567530, Sigma Aldrich
Sodium dodecyl sulfate	5%, Cat. No. L3771, Sigma Aldrich
MDA standard	Cat. No. 10009202, Cayman Chemical
Ice	
Redistilled water	
Disposable gloves	
Pipette tips	
96-well plate	Clear
15 mL tubes	
Eppendorf tubes	

METHOD DESCRIPTION:

Thiobarbituric acid reactive substance (TBARS) assay is another method to detect lipid oxidation. This assay measures malondialdehyde (MDA), which is a split product of an endoperoxide of unsaturated fatty acids resulting from oxidation of lipid substrates. The MDA reacts with thiobarbituric acid (TBA) forming a pink chromogen (TBARS), which is measured at 532–535 nm. The extent of oxidation can be measured spectrophotometrically. The addition of any antioxidant moiety to the test solution inhibits the oxidation process, and the reduced chromogen formation indicates the antioxidant capacity. The result is quantified with a calibration curve using MDA or in term of percentage inhibition.

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ARRAY DESCRIPTION:

1. Prepare 0.53% TBA solution in 20% acetic acid. Check pH and adjust to 3.5 using NaOH.

2. Dilute 250 µL MDA standard with distilled water to 1 mL. This is your MDA stock solution. Use the stock solution and distilled water to prepare an array of standards containing 0-50 µmol/L MDA. The standards will serve to build the MDA standard curve.

3. Add 100 µL sample or standard into a 15 mL conical tube.

4. Add 100 µL SDS and mix well.

5. Add 4 mL TBA and mix well.

6. Boil the samples in a water bath at 100°C for 1 hour.

7. Place the samples on ice for 10 minutes to stop the reaction.

8. Centrifuge the samples at 3 800 RPM and 4°c for 10 minutes.

9. Place 150 µL of the resulting supernatants into 96-well plates in duplicates.

10. Measure the absorbance at 530-540 nm.

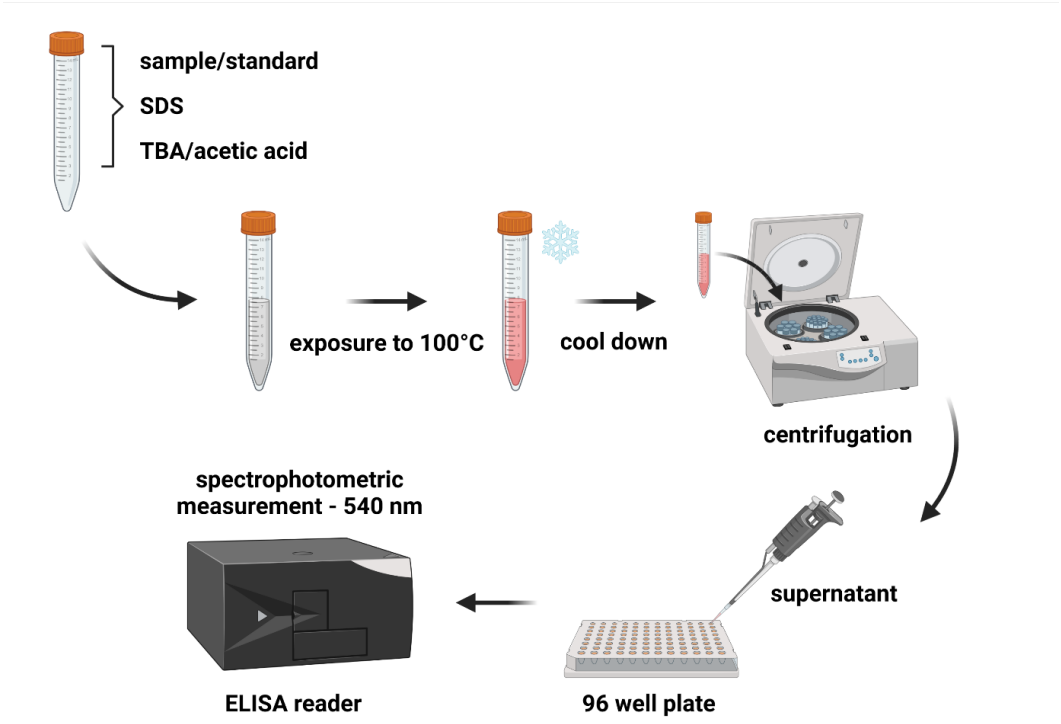

Fig. A simple summary of the technique

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INTERPRETATION OF THE RESULTS:

Calculate the results from the standard curve constructed from the absorbances corresponding to the standards used in the assay.

LITERATURE:

1. Kumar, S., Chaitanya, R.K. and Preedy, V.R. 2018. Assessment of Antioxidant Potential of Dietary Components. In: HIV/AIDS. Oxidative Stress and Dietary Antioxidants. Editor(s): Victor R. Preedy, Ronald Ross Watson. Academic Press, pp. 239-253.
2. Tvrdá, E., Debacker, M., Ďuračka, M., Kováč, J. and Bučko, O. 2021. Quercetin and Naringenin Provide Functional and Antioxidant Protection to Stored Boar Semen; Animals, Vol. 10, p. 1930. https://doi.org/10.3390/ani10101930 Cayman Chemical.
3. TBARS assay kit protocol. https://cdn.caymanchem.com/cdn/insert/10009055.pdf

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26. Total antioxidant capacity (TAC)

EQUIPMENT AND REAGENTS:

Equipment and reagents - not supplied with the kit	
Equipment	Description
96-well Elisa Plate Reader	Cat. no 51119000, Multiscan
Micropipettes	Range from 10 µL to 1000 µL
Reagent and Materials	
Samples	
Antioxidant Assay Kit	Item No. 709001, Cayman Chemical
Distilled water	
Disposable gloves	
15 mL tubes	
Sterile tips	
96 well plate	Clear

METHOD DESCRIPTION:

The combined antioxidant activities of all the constituents of a biological sample including vitamins, proteins, lipids, glutathione, uric acid, etc. are assessed in this protocol. The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS® (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS®•+ by metmyoglobin. The amount of ABTS®•+ produced can be monitored by reading the absorbance at 750 nm or 405 nm. Under the reaction conditions used, the antioxidants in the sample cause suppression of the absorbance at 750 nm or 405 nm to a degree which is proportional to their concentration. The capacity of the antioxidants in the sample to prevent ABTS® oxidation is compared with that of Trolox, a water-soluble tocopherol analogue, and is quantified as millimolar Trolox equivalents.

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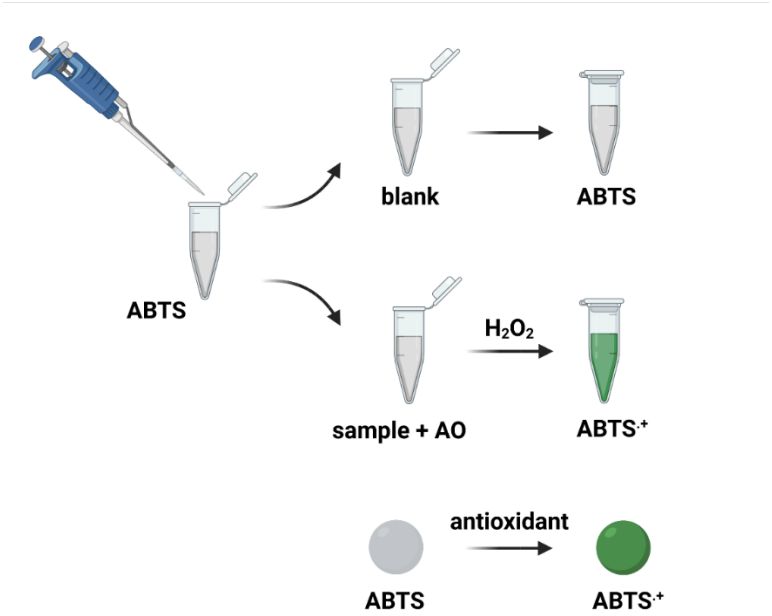

Fig.1 Principle of the method

ARRAY DESCRIPTION:

Cayman's Antioxidant Assay can be used to measure the total antioxidant capacity of plasma, serum, urine, saliva, or cell lysates.

1. All reagents and samples are equilibrated to room temperature at least 30 min prior to the beginning of the assay. Trolox standards and reagents are prepared as per the manufacturer's instructions at the time of the assay.

2. The antioxidant assay buffer concentrate is diluted 1:9 in a 15 mL conical tube. The reconstituted vial is stable for 6 months when stored at 4 °C.

3. The lyophilized metmyoglobin powder is reconstituted with 600 µL of assay buffer and is adequate for 60 wells. The reconstituted reagent is stable for 1 month when stored at −20 °C.

4. One mL of reconstituted lyophilized Trolox is used to prepare the standard curve. It is stable for 24 h at 4 °C.

5. The working solution of 441 µM is prepared by two serial dilutions, first by adding 10 µL of 8.82 M hydrogen peroxide to 990 µL of ultrapure water and then further by removing 20 µL and diluting with 3.98 mL of ultrapure water. It is stable for 4 h at room temperature.

6. Chromogen (containing ABTS) is reconstituted with 6 mL of ultrapure water which is sufficient for 40 wells. The reconstituted vial is stable for 24 h at 4 °C. It is light sensitive and was prepared in indirect light.

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7. All samples are diluted 1:9 with assay buffer in clear microfuge tubes to avoid variability because of interference by the sample proteins or sample dilution. All the samples should be analyzed in duplicate.

8. Ten µL of Trolox standard and test samples are loaded into the corresponding wells of a 96-well plate and assayed in duplicate.

9. Ten µL of metmyoglobin and 150 µL of chromogen are added to all standard/sample wells. The reaction is initiated by adding 40 µL of hydrogen peroxide as quickly as possible.

10. The plate is covered and incubated for 5 min on a horizontal plate shaker at room temperature.

11. Absorbance is monitored at 750 nm using a microplate reader.

INTERPRETATION OF THE RESULTS:

1. Determination of the reaction rate is done by calculating the average absorbance of each standard and sample. The average absorbance of the standards as a function of the final Trolox concentration (µM of Trolox equivalent) is plotted for the standard curve in each run, from which the unknown samples are determined.

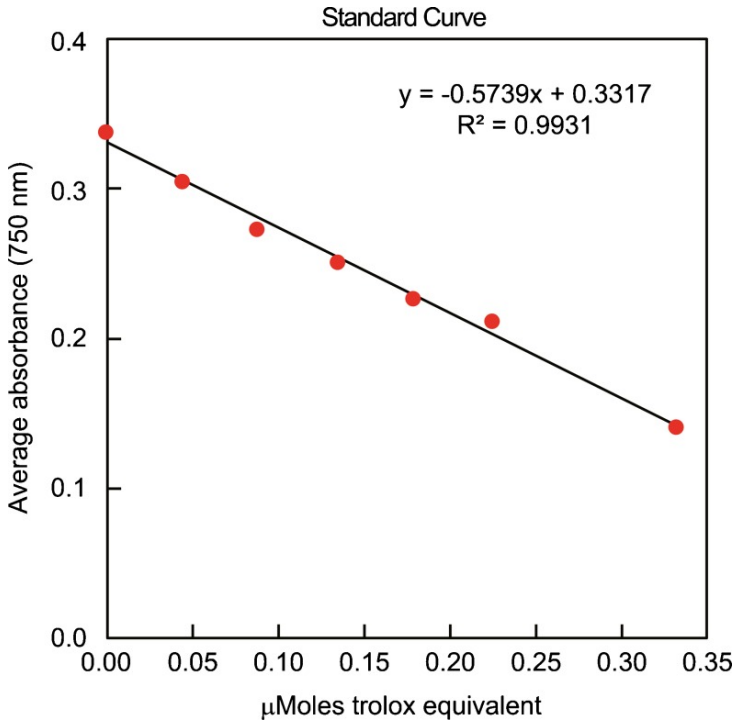

Fig.2 Example of a standard curve for TAC measurement

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2. The total antioxidant concentration of each sample is calculated using the equation obtained from the linear regression of the standard curve by substituting the average absorbance values for each sample into the equation:

Antioxidant (µM) = [(Unknown average absorbance – Y-intercept) ÷ Slope] × dilution × 1000

LITERATURE:

1. Roychoudhury, S., Sharma, R., Sikka, S., and Agarwal, A. 2016. Diagnostic application of total antioxidant capacity in seminal plasma to assess oxidative stress in male factor infertility; Journal of assisted reproduction and genetics, Vol. 33, No. 5, pp. 627–635.
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3. Cayman Chemical. 2021. Antioxidant assay kit protocol. https://cdn.caymanchem.com/cdn/insert/709001.pdf

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27. Transmission electron microscopy

EQUIPMENT AND REAGENTS:

Equipment, reagent and materials	
Transmission electron microscope	JEM100CXII, Jeol, Japan
Fixative solution	2% paraformaldehyde and 2.5% glutaraldehyde in 0.15 mol/L sodium cacodylate buffer, pH 7.1–7.3
Ultramicrotome	Leica Microsystems, Bratislava, Slovakia
Cacodylate buffer	0.15M
Osmium tetroxide	
Agar (2%)	DIFCO, Becton Dickinson, USA
Acetone series	
Poly/Bed resin	Polysciences Inc., Warrington, USA
Uranyl acetate	5%
Lead citrate	3%
Disposable gloves	
Micropipets	

METHOD DESCRIPTION:

First, semen samples collected from rams is fixed in fixative solution (2% paraformaldehyde and 2.5% glutaraldehyde in 0.15 mol/L sodium cacodylate buffer, pH 7.1–7.3) during 1 hr at 4°C. Subsequently, the semen is washed three times in cacodylate buffer for 15 min. Sperm pellets are post-fixed in 1% osmium tetroxide in cacodylate buffer during 1 hr and embedded into 2% agar (DIFCO, Becton Dickinson, USA). Subsequently, samples are dehydrated by passing through an acetone series (50, 70, 95 and 100%) and embedded into Poly/Bed resin (Polysciences Inc., Warrington, USA). Blocks of semen were cut into ultrathin sections (70 nm) using a ultramicrotome (Leica Microsystems, Bratislava, Slovakia). Ultrathin sections are placed on nickel grids, contrasted with uranyl acetate and lead citrate and examined on a transmission electron microscope (JEM100CXII, Jeol, Japan) (Fig. 1) operating at 80 kV. For each group of examiened samples, electronograms are made at the magnification × 10,000. Randomly selected view fields are evaluated on multiple meshes with ultrathin section. From each group, at least 400 individual sperm are evaluated for chosen property.

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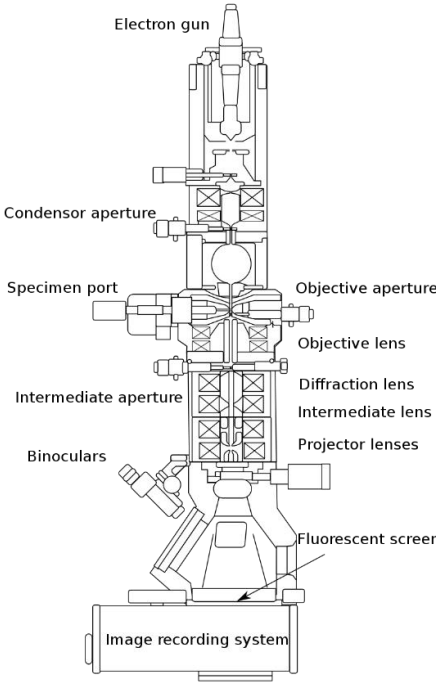

Fig. 1. Scheme of the standard transmission electron microscope (TEM)

INTERPRETATION OF THE RESULTS:

The results of this method are the image outputs of the cell ultrastructure, cell organelles, etc. These can be used, for example, as material for analyzing of the ultrastructural changes that regularly occurs in cells or the damage caused by the cryopreservation process (acrosome status of cryopreserved spermatozoa) (Fig.2).

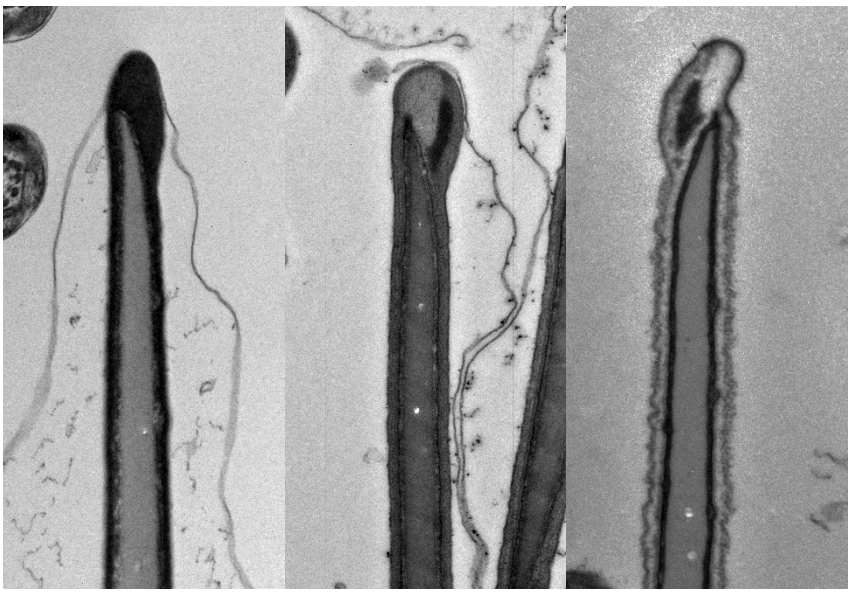

Figure 2: Intact, swollen and demaged acrosome of cryopreserved ram spermatozoa

DOI: https://doi.org/10.15414/2023.9788055226248

LITERATURE:

1. Svoradova, A., Kuzelova, L., Vasicek, J., Olexikova, L., Balazi, A., Kulikova, B., Hrncar, C., Ostro, A., Bednarczyk, M., Chrenek, P. 2018. The Assessment of Cryopreservation on the Quality of Endangered Oravka Rooster Spermatozoa Using Casa and Cytometry. Cryo Letters. 39(6), pp. 359-365. PMID: 30963152.11.2021)
2. Olexikova, L., Miranda, M., Kulikova, B., Baláži, A., Chrenek, P. 2019. Cryodamage of plasma membrane and acrosome region in chicken sperm. Anatomia Histologia Embryologia. 48(1), pp. 33-39. doi: 10.1111/ahe.12408. Epub 2018 Oct 17. PMID: 30334273.

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28. Triple assay protocol for cell viability, metabolic activity, membrane integrity and lysosomal integrity

EQUIPMENT AND REAGENTS:

Equipment and reagents - not supplied with the kit	
Equipment	Description
96-well Elisa Plate Reader	cat. no 51119000, Multiscan
CO2 incubator	Adjustable to 37 °C
Digital Mini Rotator	cat. no 88882007, Thermo Scientific
Micro pipettes	Range from 100 µL to 300 µL
Optional equipment	
Equipment	Description
Multichannel Pipette	Range from 100 µL to 300 µL
Reagent and Materials	
AlamarBlue solution	Sterile, ultrapure water
MEM – Minimum Essential Media	5-Carboxyfluorescein Diacetate, Acetoxymethyl Ester - solution
Multichannel Pipette Reservoir	Fluorescence Optical Kit, Red (Ex 625 nm, Em 660-720 nm)
96 well plate	Fluorescence Optical Kit, Green (Ex 525 nm, Em 580-640 nm)
Disposable gloves	Fluorescence Optical Kit, Blue (Ex 490 nm, Em 510-570 nm)
Sterile filter tips	Neutral red solution
15- and 50-ml tubes	Lysis soluton (1% acetic acid + 50% Ethanol)
Dulbecco's Phosphate Buffered Saline (DPBS)	

METHOD DESCRIPTION:

Cell viability is defined as the number of healthy cells in a sample. The measurement of cell viability plays an important role for all forms of cell culture. Sometimes it is the main purpose of the experiment as in toxicity assays, or it can be used to correlate cell behavior to the number of cells.

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This combination of methods is based on three independent viability tests measured simultaneously in one well of 96-well plate. We monitor three different mechanisms of action of exposure agents on the monitored cells in *in vitro* conditions. Specifically, this assay includes 3 tests.

1. Test evaluating the metabolic activity of cells (AlamarBlue – AM).
2. Test that determines the integrity of cell membrane (5-Carboxyfluorescein Diacetate, Actocymethyl Ester- CDFDA-AM).
3. Test to monitor the integrity of cell lysosomes using their ability to accumulate another type of dye (Neutral Red- NR)

ASSAY PROTOCOL:

1. Make working solution for AB/CFDA-AM test (dark room). Add 6,7ml MEM without phenol red and supplements + 350 µL of AlamarBlue solution + 7 µL of CFDA-AM solution.
2. Discard exposure medium from well plate
3. Carefully wash the cells adhered in wells – 2x 200 µL DPBS so that we do not release cells or damage the cell monolayer.
4. In dark room – pipette 100 µL of AB/CFDA AM solution per well.
5. Incubation of cells in the CO2 incubator for 30-45 minutes.
6. After incubation, measure fluorescence AB- green filter (Ex 525nm, Em 580-640nm), CFDA-AM – blue filter (Ex 490, Em 510-570nm).
7. Meantime, prepare a working solution for Neutral red (dark room): 7ml MEM without phenolic red and supplemets +106 µL NR solution.
8. Discard AB/CFDA-AM solution from well plate.
9. Carefully wash the cells adhered in wells – 2x 200 µL DPBS so that we do not release cells or damage the cell monolayer.
10. In dark room – pipette 100 µL of NR solution per well.
11. Incubation of cells in the CO2 incubator for 1 hour.
12. After incubation – discard NR solution from well plate, wash 2x by 200 µL of DPBS carefully, so that we do not release cells or damage the cell monolayer.
13. Pipette 100 µL of lysis solution (1% acetic acid + 50% ethanol) to each well and leave the plate on lab shaker for 30 minutes.
14. Measurement of neutral red – red filter (Ex 625, Em 660-720nm).

DATA ANALYSIS:

Cell proliferation assays

1. Average the duplicate reading for each sample.
2. Subtract the culture medium background from your assay reading. This is the corrected absorbance.
3. The amount of absorbance is proportional to cell number.

DOI: https://doi.org/10.15414/2023.9788055226248

For cell counting, a standard curve can be established with known cell number and fixed incubation times with the assay reagent.

LITERATURE:

1. Kamiloglu, S., Sari, G., Ozdal, T. and Capanoglu E. 2020. Guidelines for cell viability assay; Food Frontiers, Vol. 1, No. 3, pp. 332-349.

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29. 2D electrophoresis

EQUIPMENT AND REAGENTS:

Equipment	
Equipment	Description
Mini-PROTEAN tetra cell system	Biorad
Vertical electrophoretic unit	Hoefer SE 600 Chroma
Pipettes	Range from 1 µl to 1000 µl
Reagent and Materials	
EDTA	SIGMA
Tris-HCl	SIGMA
Mercaptoethanol	SIGMA
Phenol	SIGMA
Ammonium acetate	SIGMA
Methanol	SIGMA
Acetone	SIGMA
Ethanol	SIGMA
Urea	SIGMA
CHAPS	SIGMA
Dithiotreitol (DTT)	SIGMA
Ampholyt	SIGMA
SDS	SERVA
Glycerol	SIGMA
Iodoacetamide	SIGMA
SERVA IPG BlueStrips	SERVA
Gloves	
Sterile filter tips	From 1 µ do 10mL
Microtubes	1.5ml microtubes

METHOD DESCRIPTION:

Two-dimensional (2-D) electrophoresis is a powerful in its ability to separate hundreds to thousands of products simultaneously. This technique uses two different electrophoretic separations, isoelectric focusing (IEF) and SDS-PAGE, to separate proteins according to their isoelectric point (pi) and molecular weight. The identities of individual protein spots from the gel can then be identified by mass spectrometry (MS) of their tryptic peptides. Together with computer assisted image evaluation systems for comprehensive qualitative and quantitative examination of proteomes, proteome analysis also allows cataloguing and comparison of data among groups of researchers.

DOI: https://doi.org/10.15414/2023.9788055226248

ASSAY PROTOCOL:

Phenolic extraction of proteins

Extraction procedure:

1. Mechanical homogenization of 0,1 g sample
2. Extraction solution 200 µl
3. Tris phenolic solution 200 µl
4. Shaking 0 °C 30 min
5. Centrifugation at 4 °C for 10 min at 5000 rpm
6. Take phenolic phase to new tube
7. 0,1 M ammonium acetate in methanol 1 ml
8. Shake 1 min
9. Protein precipitation 8 h at - 20 °C
10. Centrifugation at 4 °C for 10 min at 5000 rpm
11. Discard supernatant
12. Pelet washing 2x 80 % acetone in 1 ml
13. Centrifugation 2x at 4 °C for 10 min at 5000 rpm
14. Pelet washing 70 % ethanol in 1 ml
15. Centrifugation at 4 °C for 10 min at 5000 rpm
16. Discard supernatant

2 D Electrophoresis

2D electrophoretical procedure

1. Isoelectric focusing

a) Use extracted sample
b) Add isoelectric buffer 200 µl
c) Shake at 4 ℃ for 30 min
d) Sonificate at 20 ℃ for 1 min
e) Vortex
f) Sample applicate on isoelectric gel strip
g) Rehydrate at laboratory temperature for 1 hour
h) Strip applicate for isoelectric focusing according to the manufacturer's manual
i) Set isoelectric focusing programme for active rehydration for 12 hour at 50 V

Rapid	0,5 hour	250 V
Gradual	2 hour	5000 V
Rapid	43000 Vh	10000V
Hold	infinity	1000 V

2. Gel strip processing

a) Shake with DTT equilibration buffer in 5 ml for 15 min.
b) Shake with iodoacetamide equilibration buffer in 5 ml for 15 min

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3. SDS PAGE procedure

a) Prepare according to SDS PAGE modified protocol
b) Prepare SDS PAGE separating gel
c) Applicate of gel strip on the top of separtating gel
d) Pouring 1 % agarose gel instead of starting gel

INTERPRETATION OF THE RESULTS:

Interpretation of results is based on comparison of the separated molecules in gel according to standard sample and according to marker of molecular weight by software. The 2-DE gel images represent the 2D protein samples that appear in the electrophoreogram as dark spots, where the size of the spot depends on the amount of protein. There are a couple of pictures avaible to compare and find the differences between the proteins we are looking for.

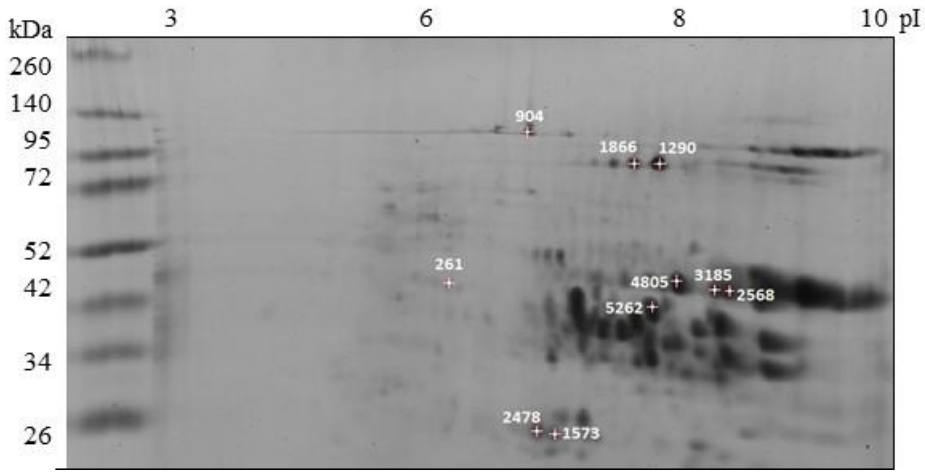

Fig.1. Protein map

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30. Western blot

EQUIPMENT AND REAGENTS:

Equipment and reagents - not supplied with the kit	
Equipment	Description
Dry Block Thermostat	Biosan SIA, Litva
Laboratory Orbital Shaker	Biosan SIA, Litva
Vortex Mixer	Thermo Scientific™
Laboratory Centrifuge	Thermo Scientific™
PowerPac™ Universal Power Supply	Bio-RAD, USA
Mini-PROTEAN® Tetra Vertical Electrophoresis Cell	Bio-RAD, USA
Trans-Blot® Turbo™ Transfer System	Bio-RAD, USA
ChemiDoc™ Imaging System	Bio-RAD, USA
Image Lab™ software	Bio-RAD, USA
Micro pipettes	Range from 2 µL to 1000 µL
Optional equipment	
Equipment	Description
Western Blot Incubation Boxes	
BCA Protein Assay kit	cat no. 23227, Thermo Scientific™
Reagent and Materials	
Samples – protein lysates in RIPA buffer	
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich
Glycine	Sigma-Aldrich
Tris Base	Sigma-Aldrich
Ultrapure water	
Sodium chloride (NaCl)	Sigma-Aldrich
1M Hydrochloric acid (HCl)	
4x Laemmli Sample Buffer	Bio-RAD, USA
β-Mercaptoethanol	Sigma-Aldrich
Precision Plus Protein™ Kaleidoscope Standard	Bio-RAD, USA
Mini-PROTEAN® TGX Stain-Free™ Precast Gel, 4-15%	Bio-RAD, USA
Trans-Blot® Turbo™ Transfer Pack	Bio-RAD, USA
Bovine Serum Albumin (BSA)	Sigma-Aldrich
Tris-Buffered Saline (TBS) 10X Stock Solution for Western Blots	Sigma-Aldrich
Tween 20	Sigma-Aldrich
1.5 ml tubes	
Primary Antibodies	

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Secondary Antibodies (HRP-conjugated antibody)	anti-mouse IgG, anti-rabbit IgG; Amersham™ ECL™, GE Healthcare, UK
Western Blotting Detection Reagent	Amersham™ ECL Select™, GE Healthcare, UK
Sterile filter tips	
Disposable gloves	

METHOD DESCRIPTION:

Western blot method is an important technique used in cell and molecular biology to separate and to identify proteins associated with many cellular processes such as cell cycle, proliferation, apoptosis, oxidative stress, cancerogenesis, inflammation, cell damage etc. By using western blotting, it is able to identify specific proteins from a complex mixture of proteins extracted from cells. The technique uses three elements to accomplish this task:

- separation by size
- transfer to a solid support
- marking target protein using a proper primary and secondary antibody to visualize.

In this technique, a mixture of proteins is separated based on molecular weight through gel electrophoresis and then transferred to a membrane producing a band for each protein. The membrane is then incubated with labels antibodies specific to the protein of interest. In the following step, the unbound antibody is washed off leaving only the bound antibody to the protein of interest. The bound antibodies are then visualized and detected by the imaging system. As the antibodies only bind to the protein of interest, only one band should be visible. The thickness of the band corresponds to the amount of protein present and can indicate the amount of protein present. It is important when quantifying protein expression to account for differences in the amount of total protein loaded into the gel using loading controls. Common loading control proteins include housekeeping proteins, such as β-actin, α-tubulin or GAPDH, quantified by Western blot, or total protein, quantified using a stain such as Coomassie Brilliant Blue or Ponceau S. A described method for total protein quantification utilizes stain-free technology and allows for protein detection on both gels and membranes.

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Bio-Rad equipment

Preparation of key solutions:

10X Running buffer: Tris/Glycine/SDS

30 g Tris base
144 g glycine
10 g SDS
Dilute in 1 L distilled water, pH should be 8.3

Loading buffer:

4x Laemmli Sample Buffer
10% and βME

Tris-buffered saline (TBS) buffer:

4.84 g Tris base
58.48 g NaCl
10 g SDS
Dilute in 1.5 L UHQ water, pH should be 7.5 (use 1M HCl). Then add 0.5 L UHQ water.

Tris-buffered saline with Tween 20 (TBST) buffer:

TBS buffer
0.1% Tween 20

Blocking buffer:

3% BSA in TBST

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ASSAY PROTOCOL:

Sample preparation

1. Remove a small volume (up to 20 µl) of lysate to perform a protein assay and determine the protein concentration foreach cell lysate samples.
2. Take 20 µg of each sample and add an equal volume of loading buffer.
3. Boil each lysate in loading sample buffer at 95°C for 5 min.
4. Vortex and centrifuge at 16,000 x g in a microcentrifuge for 1 min.

Protein separation by gel electrophoresis

1. Assemble vertical electrophoresis systems with SDS-PAGE gels and pour running buffer.
2. Load 2 µl of molecular weight markers and equal amounts of proteins (20 µg; up to 20 µl of total volume) into the wells of a mini SDS-PAGE gel (mini protean TGX stain-free gel).
3. Run the gel for 5 min at 50 V and increase the voltage to 100-150 V to finish the run in about 1 hour.
4. Capture the pre-transfer gel image and visualize protein separation using ChemiDoc imaging system such as Coomassie-like performance with no staining/destaining.

Transferring the proteins from the gel to the membrane

1. Use ready-to-use transfer packs and place the gel into PVDF membrane.
2. Assemble the transfer sandwich and make sure no air bubbles are trapped in the sandwich.
3. Place the cassette in the Trans-Blot Turbo transfer system and transfer 7 min.

Antibody incubation

1. Briefly rinse the blot in water or in TBS.
2. Block in 3% BSA in TBST at room temperature 1 hour.
3. Incubate overnight in the primary antibody solution against the target protein at 4°C. The antibody should be diluted in the blocking buffer according to the manufacturer´s recommended ratio.
4. Rinse the blot 3-5 times for 5 min with TBST in shaker.
5. Incubate in the HRP-conjugated secondary antibody solution for 1 hour at the room temperature. The antibody should be diluted in the TBST buffer according to the manufacturer´s recommended ratio.
6. Rinse the blot 3-5 times for 5 min with TBST in shaker.

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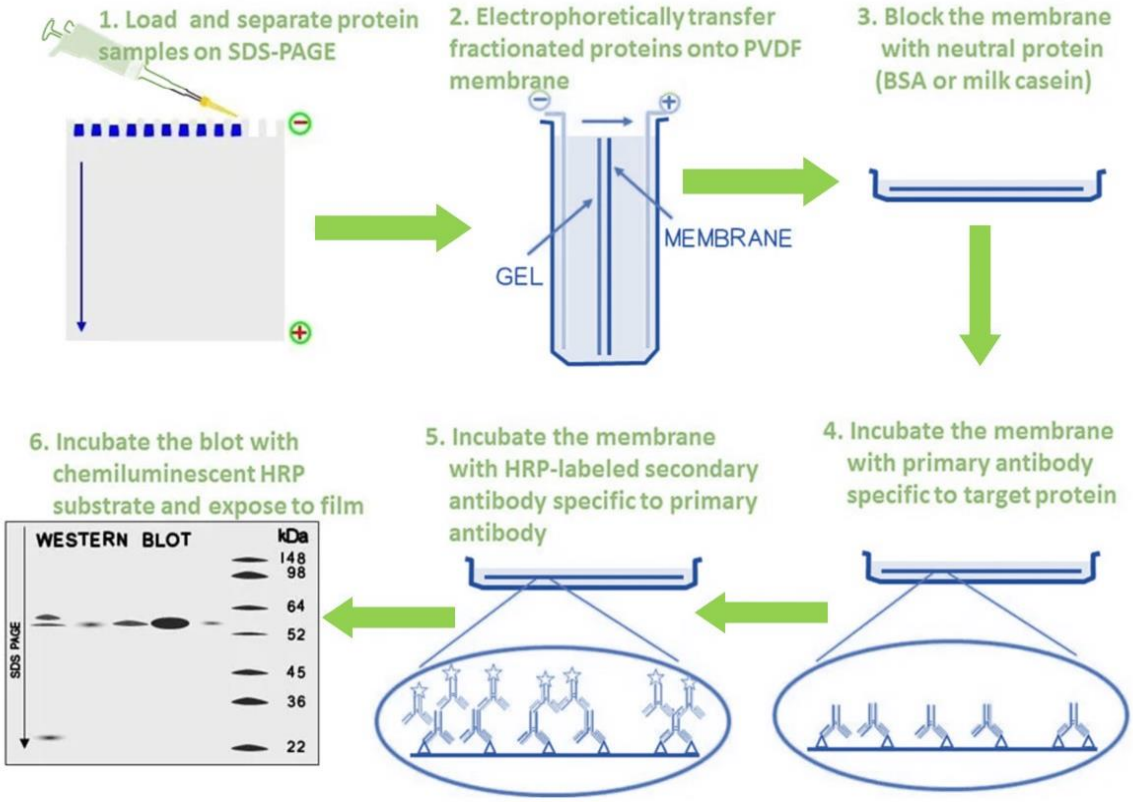
Imaging

1. Apply the Western blotting detection reagent as the chemiluminescent substrate to the blot following the manufacturer´s recommendation and incubate up to 5 min in the dark.
2. Capture the chemiluminescent signal using a camera-based imager (ChemiDoc imaging system) and save immunoblot image.

DATA ANALYSIS:

1. Use image analysis software (Image Lab software) to read and evaluate band intensity of target proteins.
2. Use the loading control protein levels to normalize the target protein levels.
3. Relative quantification expressed the level of expression/amount of target protein in the sample to the expression/amount of the same protein in the control sample.



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PARTS: III. FOOD SCIENCES and IV. FOOD TECHNOLOGY

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31. Affective sensory analysis

METHOD DESCRIPTION:

The main objective of a consumer study, or affective test, is to evaluate the personal/subjective response (preference or acceptance) of current or potential consumers towards a product or specific characteristics of the product.

It is vitally important to have the objective of the project/test perfectly defined because this type of test is extremely expensive as it involves participation of many consumers (normally above 100).

APPLICATIONS:

Development of new products: Affective tests are essential for the development of new products. For example:

- Discussion groups about the product to be made (focus group).
- Feasibility studies (direct contact with prototypes).
- Comparison with competitors' products.
- Assurance that the desired properties have not been modified in large-scale production.

Product improvement/optimization:

- ❖ To improve a product, companies try to change only one or two attributes that consumers have indicated as improvable or key ones.
- ❖ For product optimization, it is about modifying or changing the least number of possible variables (ingredients, processing stages, etc.) to obtain the desired objective (to improve product acceptance).

Practical examples of improvement/optimization are:

- Increase of a key aroma: lemon, coffee, etc.
- Increase of a texture attribute: crunchiness in dried products.
- Optimization of the roasting process due to availability of new equipment.

Market potential study: Responsibility of the Marketing department but, sensory analysts can help in the design of the questionnaire, method of conducting the tests, etc.

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TYPES OF CONSUMER STUDIES

QUALITATIVE TECHNIQUES

Focus groups (Focus panel): It is made up of a small group of 8-12 consumers, selected on the basis of specific criteria (product use, consumer demographics, etc.). They meet for 1-2 hours with the moderator of the focus group. The moderator introduces the topic of interest and facilitates discussion by using group dynamics techniques to discover as much specific information from as many participants as possible related to the focus of the session.

Mini groups, diads, triads: Mini groups, dyads and triads are an alternative to focus groups of 8-12 consumers. Mini groups are generally made up of 4-6 respondents, triads are 3 respondents, and dyads are 2 respondents with 1 interviewer. This approach is often used when it is necessary to explore into a particular discussion, if the topic being discussed is sensitive or if it is difficult to find respondents who meet the selection criteria. The format generally follows the same as a focus group.

Individual interviews: Qualitative affective tests, in which consumers are interviewed individually in a specific setting, are appropriate in situations where the researcher needs to understand and test much about each consumer or where the topic is too sensitive for a focus group. The interviewer conducts successive interviews with 12 - 50 consumers, using a similar format with each one, but investigating the responses of each consumer. Interviews or individual consumer observations can provide researchers with information about unarticulated or underlying consumer needs, and this in turn can lead to innovative products or services that meet those needs.

QUANTITATIVE TECHNIQUES

They are those that determine the responses of a large group of consumers (from 50 to several hundred, recommended around 100) to a set of questions about preferences, flavor/taste attributes, texture attributes, etc. Quantitative affective methods are applied in the following situations:

- Determine the general preference or liking (using hedonic scales, recommended of 9 points) for a producer's products by a sample of consumers representing the population for which the product is intended.
- Determine a preference or taste for broad aspects of product sensory properties (aroma, flavor, appearance, and texture).

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TYPES OF TESTS

Affective tests can be classified into two main categories based on the main test task (**Table 1**): preference or acceptance.

Table 1. Types of quantitative affective tests

	TEST GROUP	QUESTIONS
Choice	Preference tests	What sample do you prefer? Which sample do you like the most?
Classification	Acceptance Tests	How much do you like the product? How acceptable is the product?

PREFERENCE TESTS

The choice of preference or acceptance for a given affective test should be based on the objective of the project. If the project is specifically designed to compare one product directly against another in situations such as product improvement or competition parity, a preference test is indicated. The preference test forces you to choose one item over another or others. It does not indicate whether or not you like any of the products.

ACCEPTANCE TESTS

If a product researcher needs to determine the "affective state" of a product, that is, how much consumers like it, an acceptance test is the right choice. The product is compared to a well-liked company product or a competitor's. A hedonic scale is used that serves to indicate the degree of acceptability.

TYPES OF SCALES

Optimal results (more discriminatory, more actionable) are obtained with balanced scales. That is, they have an equal number of positive and negative categories and have steps of equal size. The most widely used scales are those of 5, 7 and 9 points (**Figure 1**). The most common among them is 9 points scale.

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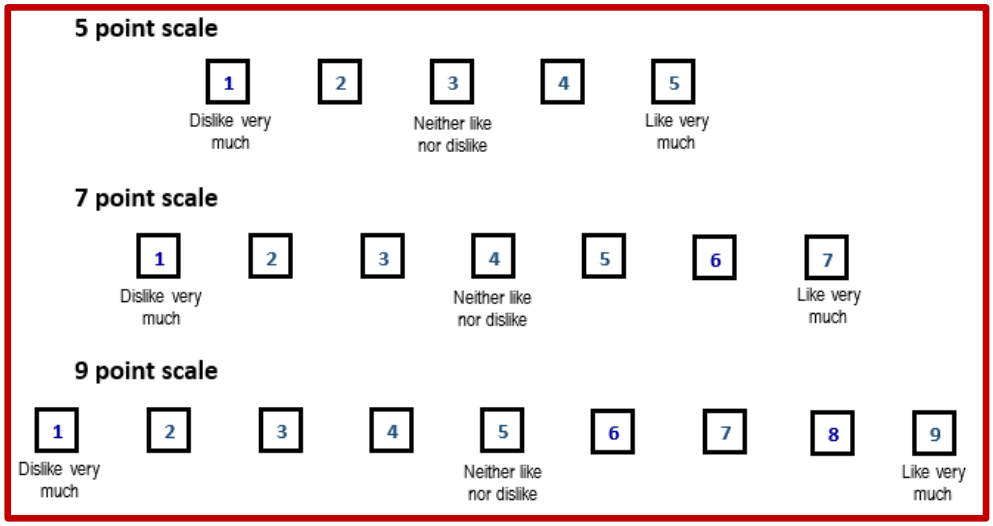

Fig. 1. Examples of scales in acceptance tests.

JAR (Just About Right)

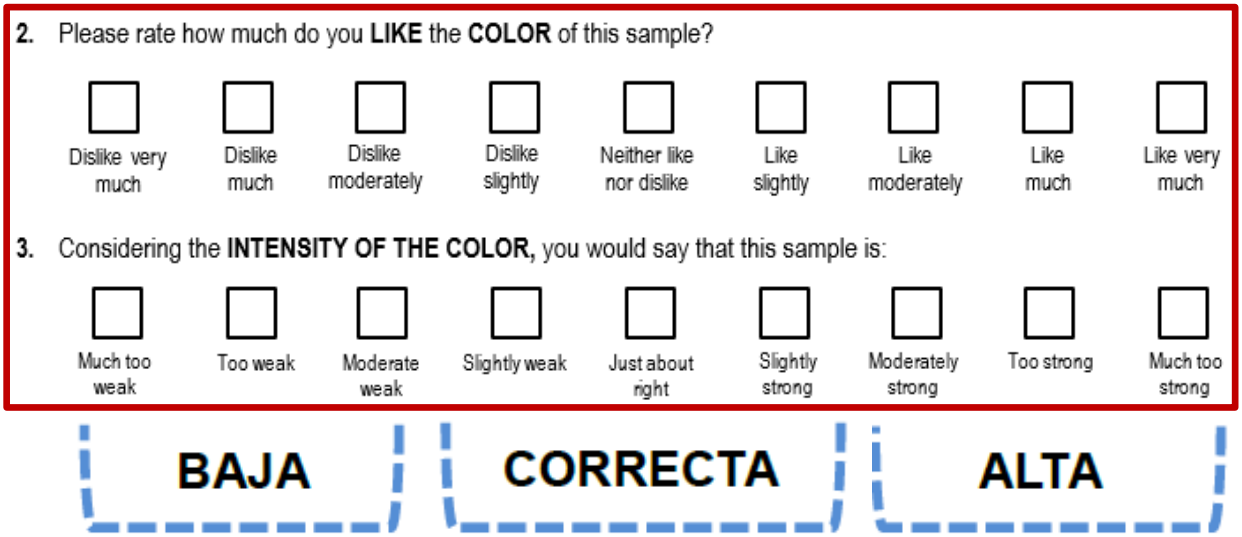

Fig. 2. Example of a 9-point acceptance scale and JAR scale of a questionnaire

There are other types of scales that complement the information that the previous ones can give. JAR (Just About Right) scales are used in consumer research to identify whether an attribute is present in a product at too high or too low level or if it is "correct". For example, to determine a consumer's preferred level of sweetness in a soft drink, consumers may be asked to test a prototype formulation and rate its sweetness on a scale ranging from "too high" to "too low" (**Figure 2**). Consumer responses will provide an indication of whether there is an opportunity to improve the prototype and suggest the direction for any potential

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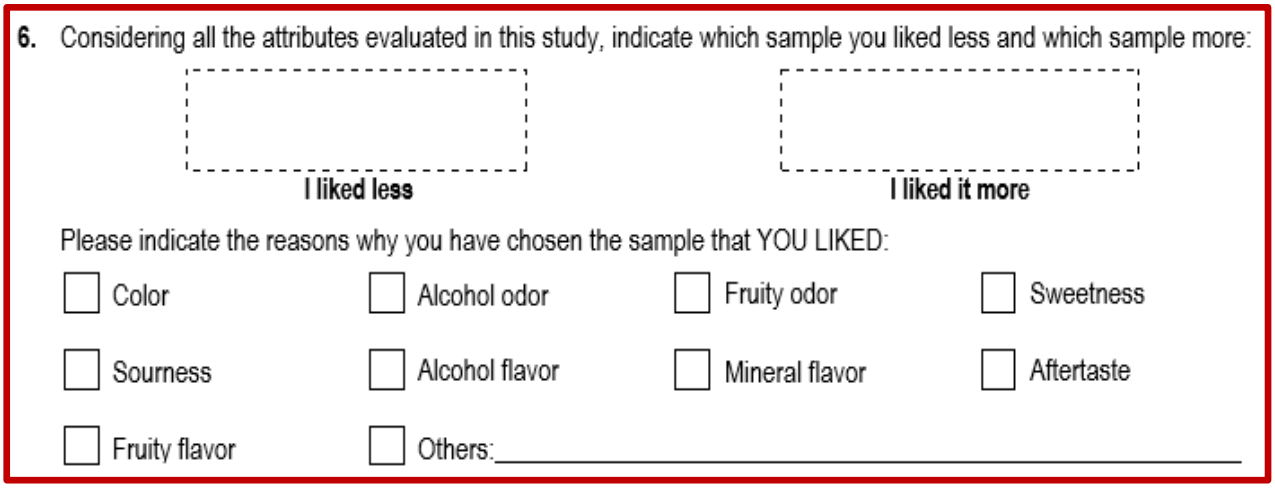
formulation changes. However, before recommending formulation changes to address an apparent deficiency, it is important to establish a link between attribute performance and overall product performance, measured by liking or purchase intent, because some attributes may not be important drivers of overall product performance.

CATA (Check-All-That-Apply)

Check All That Apply is a question format that has been used in recent years to get quick product profiles from consumers. Consumers are presented with a list of attributes and asked to indicate which words or phrases adequately describe their experience with the sample that is being evaluated. Terms can include sensory attributes, as well as hedonic responses, emotional responses, purchase intentions, potential applications, product positioning, or other terms that the consumer may associate with the sample (**Figure 3**). In this type of question, there are multiple options to choose. In general, emotion words are regularly presented in CATA format.


6. Considering all the attributes evaluated in this study, indicate which sample you liked less and which sample more:

I liked less

I liked it more

Please indicate the reasons why you have chosen the sample that YOU LIKED:

☐ Color ☐ Alcohol odor ☐ Fruity odor ☐ Sweetness

☐ Sourness ☐ Alcohol flavor ☐ Mineral flavor ☐ Aftertaste

☐ Fruity flavor ☐ Others:_____

Fig. 3. Example of a CATA question included in a wine study.

To relate CATA results to consumer acceptance, CATA studies are often complemented with questions of liking and/or may include the evaluation of an ideal (hypothetical) product. The CATA questions could be further combined with demographic questions (**Figure 4**). These types of questionnaires are essential on many occasions for companies, because they can reflect consumers' priority for their product from a target audience that had not been raised. Finally, questions about consumer willingness to buy the product or how much they are willing to pay for a particular product are often added at the end of an affective questionnaire.

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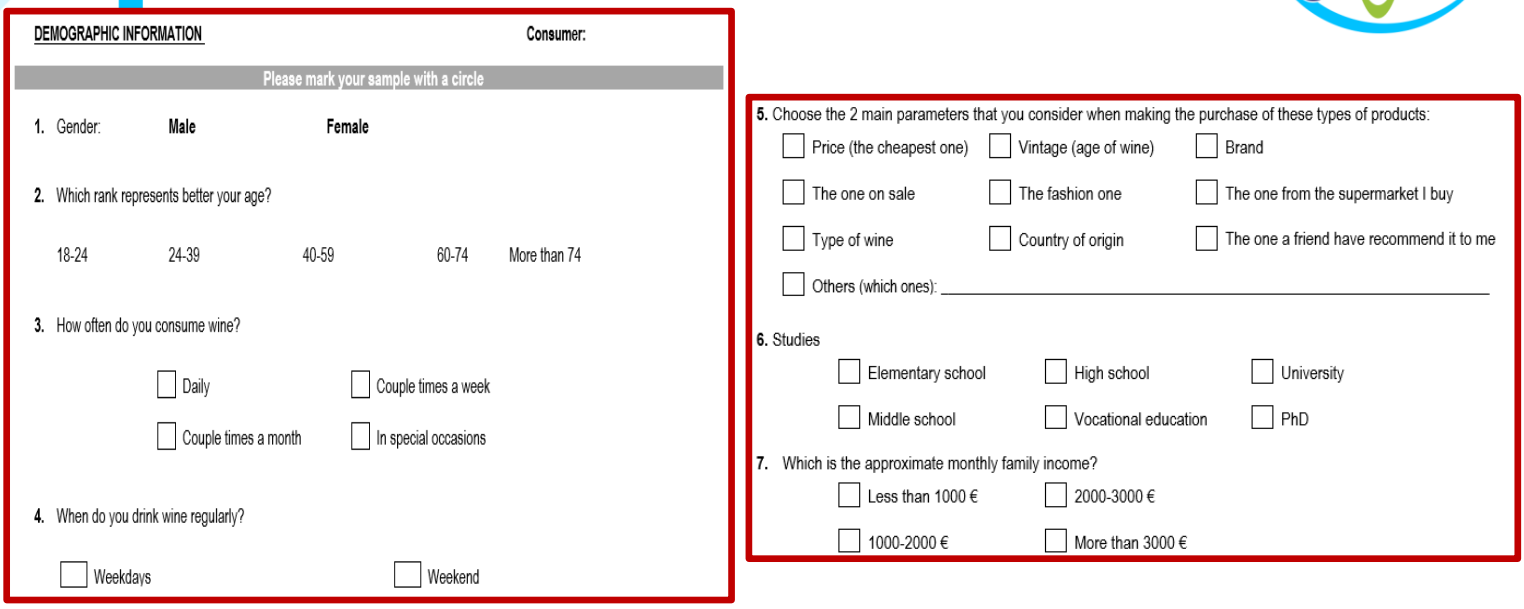

DEMOGRAPHIC INFORMATION Consumer:

Please mark your sample with a circle

1. Gender: **Male** **Female**

2. Which rank represents better your age?

18-24 24-39 40-59 60-74 More than 74

3. How often do you consume wine?

☐ Daily ☐ Couple times a week

☐ Couple times a month ☐ In special occasions

4. When do you drink wine regularly?

☐ Weekdays ☐ Weekend

5. Choose the 2 main parameters that you consider when making the purchase of these types of products:

☐ Price (the cheapest one) ☐ Vintage (age of wine) ☐ Brand

☐ The one on sale ☐ The fashion one ☐ The one from the supermarket I buy

☐ Type of wine ☐ Country of origin ☐ The one a friend have recommend it to me

☐ Others (which ones): _____

6. Studies

☐ Elementary school ☐ High school ☐ University

☐ Middle school ☐ Vocational education ☐ PhD

7. Which is the approximate monthly family income?

☐ Less than 1000 € ☐ 2000-3000 €

☐ 1000-2000 € ☐ More than 3000 €

Fig. 4. Example of demographic and willingness to buy questions included in a wine study.

STEPS TO CARRY OUT A CONSUMER STUDY

CHECK LIST

Before conducting a consumer study, the following points/issues should be considered:

- The purpose of the study.
- Type of products to compare.
- Number and quantity of samples.
- Completion of the questionnaire.
- Number of consumers/participants.
- Time and place of the study.
- Announcement/recruitment.
- Sample presentation temperature.
- Order of presentation of samples.

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QUESTIONNAIRE DESIGN

To design a questionnaire, it is recommended:

- ❖ Ask the minimum number of questions to achieve the project objective; so that respondents do not get tired and are fully concentrated during the test period.
- ❖ Keep questions clear and similar in style. Use the same type of scale, e.g., preference, hedonic, JAR, or intensity scale, within the same section of the questionnaire. The intensity and hedonic questions can be asked in the same questionnaire, but they must be clearly distinguished. The questions and your answers should follow the same general pattern in each section of the questionnaire. For consistency and to ensure accurate responses, scales should be designed to go in the same direction, for example, [Too little ····· Too much], for each attribute, so respondent does not have to stop and decode each question.
- ❖ Lead the questions to address the main differences between the products in the test. Attribute questions should relate to the attributes that are key/essential in the products under analysis and that differentiate them. Subjects/consumers will not give clear answers to questions about attributes that they cannot perceive or about differences they cannot detect.
- ❖ Ask questions only about attributes which are actionable (attributes which can be manipulated during the product processing or preparation).
- ❖ Use scales as simple as possible but ensuring proper differentiation among samples.
- ❖ Do not ask two questions in one, e.g., was the prepared food tasty and easy to cook?
- ❖ Keep the questions within the capabilities of the respondents by avoiding questions that require considerable memory or technical understanding.
- ❖ Do not allow hypothetical questions, e.g., if you had a pet, would you feed it like this?
- ❖ Ensure reverse translation of questionnaires translated into other languages to guarantee meaning is preserved.

JUDGES/CONSUMERS

Basic rules that the consumer must have:

- Good health.
- Do not wear perfumes, makeup, etc.
- Not having smoked, or consumed strong products such as coffee, at least 1 hour before the session.
- Read the questionnaire carefully.
- Tell the person in charge of the study if he/she is allergic to the product under study.

The panel must be made up of at least 100 habitual consumers of the product under study.

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PLACE OF TESTING

These tests are carried out in specific tasting rooms (**Figure 5**). These are made up of booths separated by a side wall to prevent each consumer from being influenced by other participants during the test.

Fig. 5. Tasting room.

The **room temperature** in the tasting room should be around 22 ± 2 ºC, in order to create an ideal environment for carrying out the tasting. White **lights** are used so that they cannot interfere when evaluating the color of the sample, but there must also be another type of light in case the color of the products needs to be masked. During the tastings the judges have 2 glasses, one with water to rinse their mouths between samples and another with breadsticks, or with green apple (if the food has fat) or coffee beans (if consumers are judging smell attributes, e.g. oil tasting) since these help to better clean the nose, which allows them to taste the samples as if it were always the first time.

It must also have separate areas for storage, sample preparation (cooking, heating, cooling etc.) and finally for plating the samples. They must be in an area very close to the tasting room.

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ANNOUNCEMENT/RECRUITMENT

A call is made previously to ensure proper number of participants. The way to do it can be via email, posters, etc. (**Figure 6**); where it must clearly state the product to be tasted, the day, time, and place where the study will be carried out.

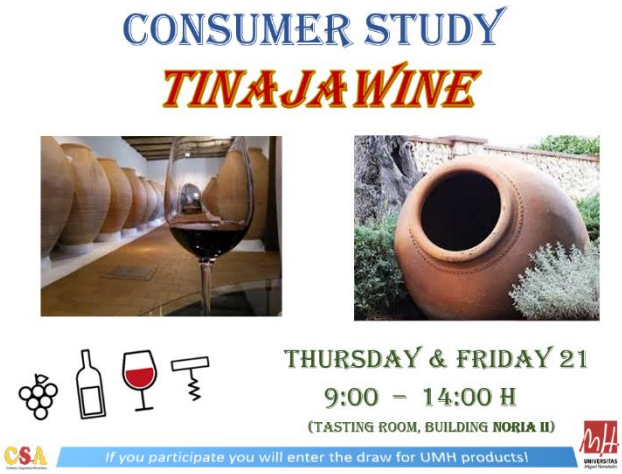

Fig. 6. Example of a poster announcement prepared for a wine study.

As a way of thanking people who carry out the tasting, they participate in a raffle where they can win prizes. Those who do not win receive a small detail such as a notebook and a pen (**Figure 7**).

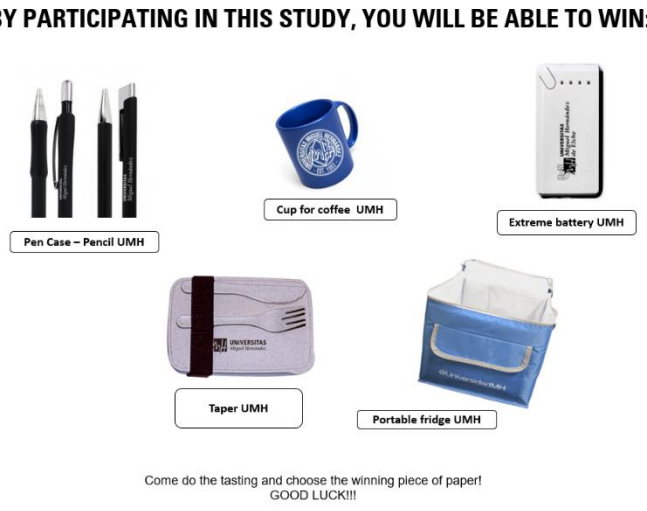

Fig. 7. Example of a poster announcement informing about rewards that can be obtained when participating in a consumer study.

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PRESENTATION AND SAMPLE SERVICE ORDER

When receiving participants in an affective test, it is important to have a control over the number of women-men participating in the study, and participants included in each age range, or any other demographic parameter considered essential for the company producing the product under study.

Samples are presented to consumers individually and in a totally random order. Each of the samples are coded with random 3-digit numbers for their identification. Each judge receives the samples and their corresponding questionnaire, previously approved by the company. The samples are always presented in the conditions as they are usually consumed or as close as possible.

It is recommended that the number of samples for each taster is between 4 and 6 for very aromatic products (smoked, spicy) or those that contain a lot of fat, because this type of food can cause sensory and mental fatigue. Temperature must be a function of the type of product and the objective of the test.

TREATMENT OF RESULTS

The results are subjected to a statistical treatment, using ANOVA, Tukey's multiple range test or LSD (among others, depending on what you want to analyze). To perform these statistical analyzes, a software called XLSTAT Premium 2016 (Addinsoft, New York, NY, USA) and StatgraphicsPlus (version 3.1, Statistical Graphics Corp., Rockville, MA, USA) can be used.

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EXAMPLE OF INFORMATION REPORTED IN RESEARCH ARTICLES:

As an example of how all this information must be summarized in a research article, here is the section "Affective Sensory Analysis" of the article entitled "Effect of aging vessel (clay-tinaja versus oak barrel) on the volatile composition, descriptive sensory profile, and consumer acceptance of red wine (Issa-Issa *et al.*, 2021).

"This affective study was conducted using 150 consumers at each of the two locations used (i) Sevilla (Spain) and (ii) Wrocław (Poland); email and flyers were used for the recruitment process. These two countries (Spain and Poland) were selected to conduct this initial study because the tradition, consumption habits, market and preferences are different. Spain is a big wine producer, but average–low consumer and Poland is a minor wine producer and average/low consuming country.

The consumer profile was as follows:

(i) Spain: 41% and 59% female and male, respectively; 34% (18–24 years old group), 44% (24 39 years old group), 20% (40–59 years old group) and 2% (60–74 years old group). Spanish consumers were used as model for wine drinkers highly accustomed to complex and intense red wine with intense oaky notes.

(ii) Poland: 44% and 56% female and male, respectively; 30% (18–24 years old group), 49% (24–39 years old group), 17% (40–59 years old group) and 3% (60–74 years old group). Polish consumers were selected considering that they are used to drink sour and fresh white wines.

The test questionnaire was developed in Spanish and, then, translated into Polish; finally, back translation from Polish to Spanish was conducted to check the proper translation. All samples were

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served in a randomized order labeled with three-digit codes. Information about wine consumption was requested to check that consumers were regular wine drinkers and agreed with the assumptions made when selecting these two countries and consumers. The information obtained was as follows (i) Spanish consumers: frequency of wine consumption: 4% daily, 33% twice a week, 22% twice a month and 41% on special occasions; regularity of drinking wine: 9% weekdays, 83% weekend and 8% all days; (ii) Polish consumers: frequency of wine consumption: 1% daily, 18% twice a week, 46% twice a month and 35% in special occasions; regularity of drinking wine: 8% weekdays, 71% weekend and 21% all days. Consumers were asked about their satisfaction degree for each of the attributes under study, using a nine-point hedonic scale (1 = dislike extremely; 5 = neither like or dislike; and 9 = like extremely). JAR questions (Just About Right) were used to ask consumers about the intensity appropriateness of each of the main wine attributes. Finally, consumers ranked samples from the least preferred one to the most preferred one. Research was approved by the ethics committee of Oficina de Investigación Responsable (Universidad Miguel Hernández de Elche, Elche, Alicante, Spain) and consumers provided their informed consent prior to participating in the study".

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32. Analysis of the organic acids and sugars profiles in foodstuffs

EQUIPMENT AND REAGENTS:

Equipment and reagents	
Equipment	**Description**
HPLC coupled with a diode-array detector (DAD) and refractive index detector (RID)	Series 1100 high-performance liquid chromatograph by Agilent
Centrifuge	Sigma 3–18 K
Filters	0.45 µm Millipore
Column and pre-column	HPLC Column: Supelcogel TM C-610H (30 cm x 7.8 mm); Pre-column: Supelguard (30 cm x 7.8 mm)
Reagent and Materials	
Reagents	
Ultra-high-purity deionized water	
Phosphoric acid 85%	
Phosphate buffer 50 mM at pH 8.5	
Standards of organic acids	citric, malic, oxalic, tartaric, etc.
Standards of sugars	glucose, fructose, sucrose, etc.
Materials	
HPLC vials	
Syringes	Up to 2 mL

METHOD DESCRIPTION:

The identification and quantification of profile of organic acids and sugars consists of two steps.

- Extraction of sugars and organic acids of the samples
- Identification and quantification by HPLC of profile of sugars and organic acids

Extraction of sugars and organic acids of the sample

0.5 g of freeze-dried (or 5 mL of juice) sample is diluted with 5 mL of phosphate buffer 50 mM at pH 7.8. Then sonicated for 15 minutes and finally centrifuged at 15,000 x g for 20 min. The supernatant is filtered through 0.45µm Millipore and stored in HPLC vials (under refrigeration for short period of time).

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Identification and quantification by HPLC of profile of sugars and organic acids

10 µL of supernatant (obtained as indicated above) is injected into a Hewlett- Packard (Wilmington DE). Series 1100 high-performance liquid chromatograph (HPLC), Agilent equipped with a refractive index detector for sugars detection, and UV/Vis detector for organic acids analysis. A column (Supelcogel ™ C-610H column 30 cm x 7.8 mm) and a pre-column (Supelguard 5 cm x 4.6 mm; Supelco, Bellefonte, PA) is used for the analyses of both organic acids and sugars. The elution (run isocratically at 30ºC) buffer consisted of 0.1% phosphoric acid at a flow rate of 0.5 mL/min and organic acids' absorbance is detected at 210 nm using a diode-array detector (DAD). These same HPLC conditions (elution buffer, flow rate and column) are used for the analysis of sugars. The detection is performed using a refractive index detector (RID).

Calibration curves, prepared with individual standards at a concentration range between 1 and 10 g L^{-1}, are used for the quantification of organic acids and sugars. Analyses are carried out in triplicate and results are expressed as mean ± standard error in g L^{-1}.

INTERPRETATION OF THE RESULTS:

Once the chromatograms of the samples (Fig. 1) are obtained, for the identification of sugars and organic acids, spectrums (organic acids) and retention times (sugars and organic acids) are employed and compared with those obtained from the chemical standards. Standard curves for pure sugars as well as for organic acids are used for quantification. The concentration of each compound is obtained by substituting the peak area (of the compound) in its respective standard curve (Fig. 2).

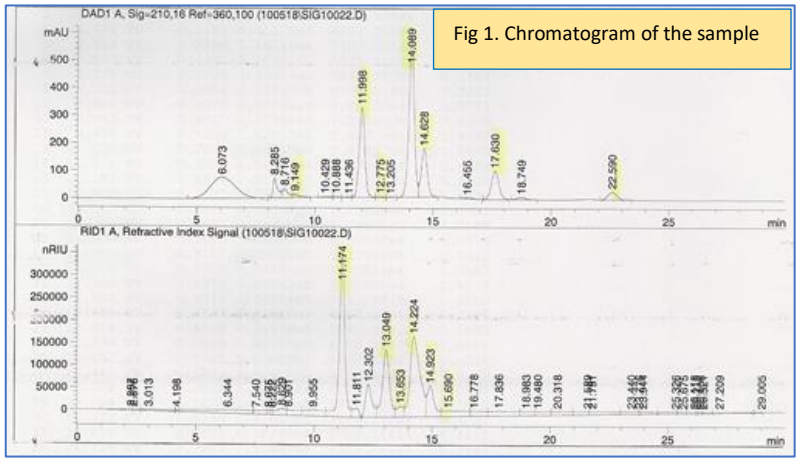

Fig 1. Chromatogram of the sample

Fig 2. Quantification

Organic acids												
		AREA (mAU)					[%] = g/100 mL					
		Phytic	Oxálic	Citric	Tartaric	Malic	Phytic	Oxálic	Citric	Tartaric	Malic	
Sample	Repetition						Sample					
								-0,124423	-0,28083	0,00742504	-0,017454	0,0745063

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33. Analysis of the fatty acid profile of food and feed

EQUIPMENT AND REAGENTS:

Equipment and reagents	
Equipment	**Description**
Gas chromatograph coupled to a Flame Ionization Detector (GC-FID)	Nexis GC2030 by Shimadzu GC-FID coupled to an automatic injector AOC20i
Shaker multiple positions	
Centrifuges	
Water bath	
Evaporation system by nitrogen flux	
Column (several available)	SP2380 Supelco 60 m, 0.25 mm internal diameter and 0.20 µm internal coating, Supelco. CP Sil 88 100m, 0.25 mm internal diameter and 0.20 µm internal coating, Agilent
Reagent and Materials	
Reagents	
Ultra-high-purity deionized water	
Hexane HPLC grade	
Dichloromethane HPLC grade	
Methanol HPLC grade	
Pyrex tubes with teflonated caps (different sizes as a function of sample)	
Glass Pasteur pipettes (long size)	
Eppendorf/tubes	
Micropipette for standards addition	
Standard for identification	37/FAME MIX by Supelco Bacterial FAME by Matreya PUFA 1 by Matreya PUFA 3 by Matreya
Reagent for derivatization (to be used depending on the method)	Sodium methoxide, Sigma Aldrich Boron trifluoride 14% methanol, Sigma
Reagent for traces of water	Sodium Sulfate anhydrous for analysis, Sigma Aldrich
Internal standards (to be used depending on the product)	Methylated C5:0, C13:0 and C19:0
Materials	
GC vials	
Eppendorf tubes	Up to 2 mL
Teflon film	

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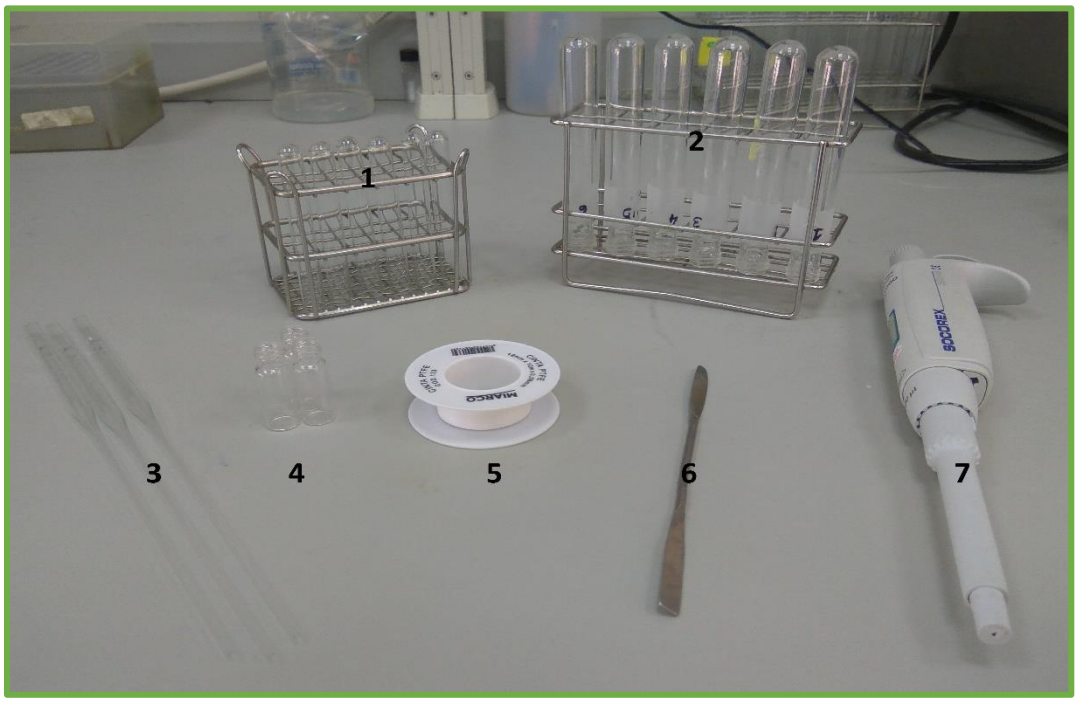
METHOD DESCRIPTION:

The identification and quantification of profile of fatty acids consists of two to three steps depending on the method.

- Fat extraction
- Hydrolysis of triglycerides and methylation of the released fatty acids. In some methodologies direct methylation with no previous extraction is suitable and recommended.
- Separation, identification and quantification by GC-FID

Fat extraction

Depending on the sample, fat needs to be previously extracted. Although several methods are available for fat extraction (Shoxlet, Folch, Dyer…), cold extraction and modified original methods to reduce solvents use are preferred. As an example, for the analysis of milk fat, we may use the Folch method with some variations as reported in Romeu-Nadal et al. (2004) and a subsequent methylation according to the method of Nudda et al. (2005)



1: Disposable glass tubes for intermediate steps; 2: Pyrex tubus with tefonate caps for centrifugation; 3: Large glass Pasteur pipettes; 4: Chromatography vials; 5: Teflon film; 6: Espatula, 7: Pipette

Following Romeu-Nadal et al. (2004): 3 mL milk are mixed with 5 ml de dichloromethane: methanol (2:1) in a centrifuge tube and further shaked for 1 minute. Afterwards tubes are centrifuged for 8 minutes at 3500 rpm at 4 °C.

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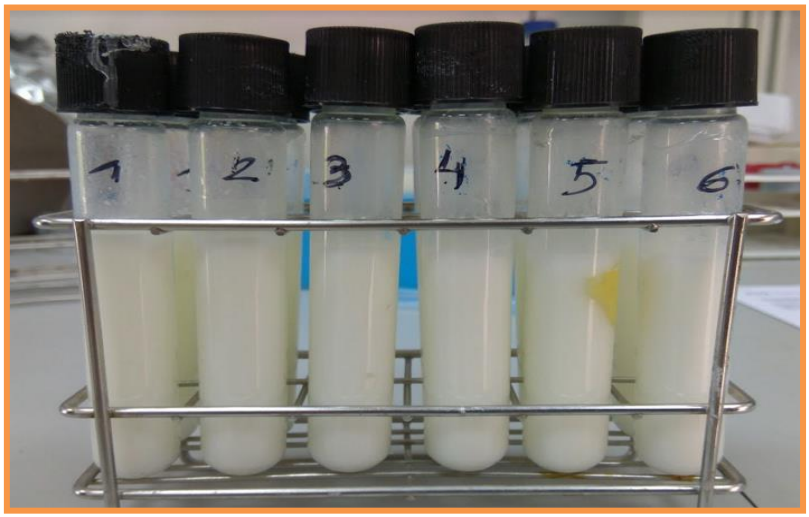

Milk mixed with solvents.

After centrifugation two layers are clearly separated: the upper aqueous layer should be gently removed, and another 3 ml of dichloromethane: methanol (2:1) added, tubes should be further shaked for 1 min and centrifuged for 6 min at 3500 rpm and 4 °C. Finally, a precipitate is formed, the organic layer should be recovered by using a large glass Pasteur pipette and transferred to another container. This extract contains the extracted fat and now it may be frozen stored or directly dried under nitrogen flow to recover fat for further derivatization.

Hydrolysis of triglycerides and methylation of the released fatty acids

Fatty acid profile analysis in diets is performed by direct methylation on the lyophilized samples, without prior extraction of the fat, according to Kramer et al. (1997). Methodology needs to be adapted to the samples, taken special consideration if standard methods apply for such samples.

Weigh a quantity of lyophilized sample containing about 12-15 mg of fat into 40 ml Pyrex glass tubes. Add 100 µl of dichloromethane and then add 1 ml of sodium methoxide while carefully mixing by hand in a circular “wrapping” motion to mix the reagent with the sample. Allow to react for 1 minute.

Keep in orbital bath at 90 °C for 10 minutes. After that time, rapidly cool to 4 °C by placing the tubes on a tray with ice up to room temperature. Once the tubes have warmed, add 1 ml of boron trifluoride 14% methanol to each tube. Next, the Pyrex tubes are placed in the dark for 30 minutes at room temperature.

Add 1 ml of distilled water. Add with 1 ml of hexane with internal standard at a concentration of 0.5 mg/ml. Shake for 1 minute in a vortex and centrifuged for 10 min at 4000 rpm and 4 °C. The anhydrous sodium sulfate is deposited in eppendorf tubes. This will help absorb any

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remaining water left on the top layer. After centrifugation, the upper layer (organic phase) is extracted with a 250 mm glass Pasteur pipette and deposited in eppendorf tubes. Let sit for 3-5 minutes with the sodium sulfate anhydrous.

Collect hexane layer, transfer to chromatography vials, and inject into equipment.

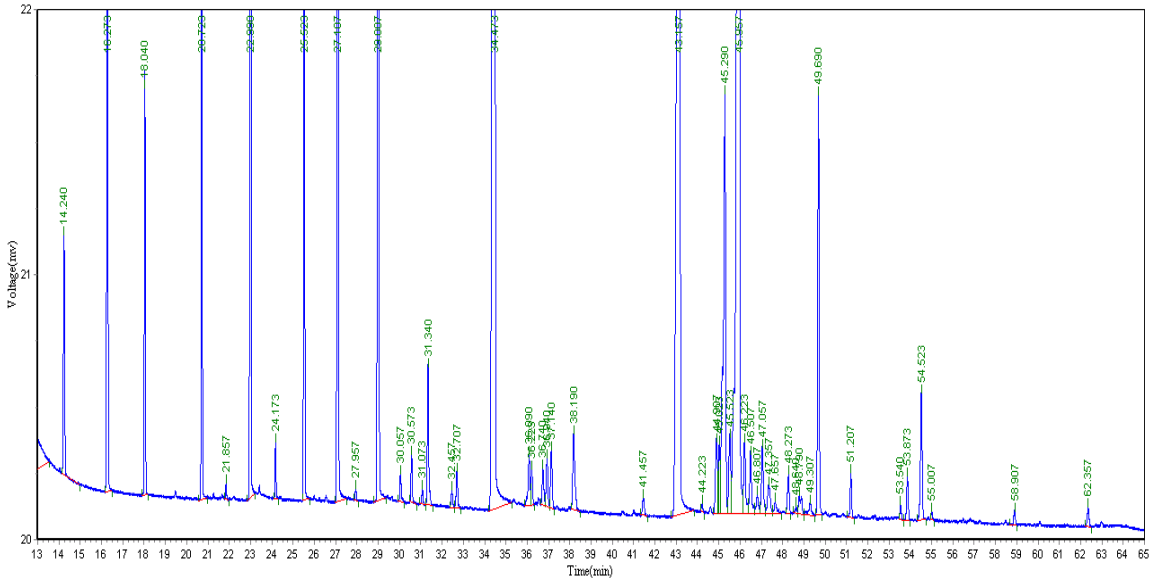
Identification and quantification of methylated fatty acids by GC-FID profile of fatty acids

1 µL of hexane extract (obtained as indicated above) is injected into a Shimadzu Nexis GC2030 by Shimadzu GC-FID. Helium is used as mobile phase, nitrogen as makeup gas (40ml/min), and synthetic air (35ml/min) and hydrogen (400ml/min) for the flame. Flux of Helium and split conditions are applied according to the method suitable for each food/feed.

Analyses are carried out in triplicate and results are expressed as mean ± standard error in g/kg feed or percentage on the total fatty acid profile, depending on the methodology used.

INTERPRETATION OF THE RESULTS:

Once the chromatograms of the samples (Fig. 1) are obtained, for the identification of fatty acids retention times of standards are taken as references for identification. If no internal standard has been used only semi-quantification is available based on the relative percentage of the area of each peak. The area of the concentration of each compound is obtained by substituting the peak area (of the compound) in its respective standard curve).



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Full profile of goat milk in CPSil 88 (C5:0 and C13:0 used as internal standards). Chromatographic conditions:

- Capillary column: CP-Sil 88, 100 m × 0.250 µm i.d., 0.20 µm film thickness, Agilent Technologies;
- Detector: Flame ionization detector (FID) set at 260°C;
- Carrier gas: Helium (1 mL/min flow rate) with a pressure of 28 psi;
- Volume of sample injected: 1µl (split ratio: 1:80, injector temperature: 250°C)
- Oven temperature programmed as follows: initial temperature set at 45°C for 4 min, increased at 13°C/min to 175°C, and held for 27 min; then it increased at 4°C/min to 215°C, and held for 35 min.

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34. Analytical method for simultaneous determination of phthalates and bisphenol A in honey samples based on Dispersive Liquid-Liquid Micro Extraction (DLLME) followed by GC-IT/MS.

EQUIPMENT AND REAGENTS:

Equipment	
Equipment	Description
Gas chromatograph (GC) TraceGC (ThermoFischer, Milan, Italy)	Separation of analytes on the basis of chemical and physical characteristics.
Ion Trap Mass Spectrometry (IT/MS) PolarisQ (ThermoFischer, Milan, Italy)	Qualitative-quantitative analysis.
Vortex ZX3	To favor the formation of the macroemulsion.
Ultrasounds Starsonic 18-35	To form the microemulsion.
Centrifuge Neya 8	Break the microemulsion.
Reagent and Materials	
Chemical standard: Dimethyl phthalate (DMP); Diethyl phthalate (DEP); Diisobutyl phthalate(DiDP); Dibutyl phthalate (DBP); Bis(2-ethylhexyl) phthalate (DEHP); Di-n-octyl-phthalate (DnOP); Bisphenol-A (BP-A). Internal standard: Phenanthrene (PHE). Solvent: Acetone; n-Heptane; iso-Octane; Toluene; Benzene. Other chemical: Sodium chloride; UP water.	

METHOD DESCRIPTION:

Preparation of standard solutions

For the development of the method and the study of the method parameters, solutions of known concentrations of the standards in question were made. Specifically, 10 mg of each

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PAE/BPA were weighed by analytical balance and solubilized in 10 mL of acetone. The preparation of the internal standard (I.S.) solution was done by weighing 1 mg of phenanthrene solubilized in 20 mL of acetone (50 µg g^{-1}).

Preparation of calibration curves

The mother solutions have been diluted with acetone to set up a PAEs/BPA mix solution. Appropriate dilution of the PAEs/BPA mix solution, 10 µg g^{-1}, with acetone eas carried out to obtain solutions of known concentrations (0.05, 0.5, 1.0, 2.5, and 5.0 µg g^{-1}).

Study of extraction solvent

All optimization procedures were carried out on blank honey sample spiked with known amounts of PAEs/BPA. A blank sample of honey was collected from a local apiary and stored in darkness at 4 °C in PAEs-free glass bottles. The study was conducted following the sample analysis procedure.

Sample analysis procedure

2.5 g of honey and 7.5 µL 50 pg $µL^{-1}$ of phenanthrene were brought to 10 g of aqueous solution, pH 4. Carefully following the safety rules, 75 µL of toluene, identified as the best extraction solvent, were added. Subsequently, the sample was subjected to 5 min stirring and ultrasounds for 6 min and NaCl 10 g L^{-1} was added. The solution was centrifuged for 30 min at 4000 rpm to break the emulsion, then 1 µL was injected into the GC–IT/MS instrument.

Study of method parameters

The analytical protocol was validated in terms of linearity range, correlation coefficients, reproducibility, intra- and inter-day errors and recoveries, and by performing the entire procedure on honey samples.

Effect of thermal stress

To verify the release of the analytes under heat stress conditions, some honey samples in disposable plastic packages were subjected to thermal stress in an oven at 40 °C for 24 h and subsequently analyzed as described in the sample analysis procedure.

INTERPRETATION OF THE RESULTS:

The search of PAEs and BP-A was conducted using gas chromatography coupled to mass spectrometry. The latter allowed for a better cleaning of the chromatogram through the acquisition of data in SIM and the confirmation of the analyte through the database. Quantification was performed via calibration curves using the internal standard. The results were expressed as required by Reg UE n. 10/2011 and Reg UE n.213/2018.

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DEVICE PERFORMANCE

The analytical instrumentation supplied in our laboratory is constantly updated. All the tools are automated and require the use of specific software and qualified personnel.

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35. Descriptive sensory analysis - expert panel

METHOD DESCRIPTION:

Sensory analysis is an important tool for consumer-driven development, production and distribution of food products with consistently high sensory quality.

To implement the sensory quality of a product and develop a method for its evaluation, it is very important to know the sector involved. This implies knowing the type of company, the production systems and the variability of the product and thus, being able to gather scientific and market information that is related to the type of product under study. A sensory analysis panel is a measuring instrument that employs people to carry out determinations. For them, it will be essential to consider very well in advance all the tests and exercises to be carried out during it, as well as the use that it will have once it is formed.

Descriptive sensory analysis is one of the most important sensory analysis methodologies. The main objective of this analysis is to find descriptors that contain a maximum of information about the sensory characteristics of the product under study.

FIELDS OF APPLICATION

Descriptive tests are one of the most important sensory analysis methodologies. The main objective of this analysis is to find descriptors that contain a maximum of information about the sensory characteristics of the product under study, such as aroma, flavor and texture.

Descriptive sensory analysis applications include:

- To develop or change a product.
- To define a product, standardize production and marketing processes in terms of its sensory attributes.
- To study and improve product lifespan.
- To compare a product with a pattern or with other similar products on the market or under development.
- To prepare the map of the perceived attributes of the product in order to relate them to factors such as instrumental, chemical or physical properties, and / or consumer acceptability.

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COMPONENTS OF THE DESCRIPTIVE ANALYSIS

The components of the descriptive analysis include:

QUALITATIVE ASPECT

This aspect is focused on the sensory characteristics that define a product. These are called: attributes, notes, character, descriptive terms or descriptors. Among these characteristics we have: (*i*) appearance, (*ii*) aroma, (*iii*) oral texture, (*iv*) skin texture. The set of these qualitative aspects constitutes the product footprint.

The success of this aspect lies in:

- Selection of terms based on the technical and physiological principles of flavor, texture or appearance.
- Training of judges to understand and use terms consistently.
- Use of reference samples for terminology to ensure consistent application of descriptive terms.

QUANTITATIVE ASPECT

This aspect is based on the intensity of the attributes, which indicate the degree to which each of these attributes is present in the sample under study. This intensity is expressed by assigning a value using a measurement scale.

The success of this measure depends on:

- Selection of a suitable scale.
- Training of judges to use the selected scale consistently across all samples and over time.
- Use of reference samples to ensure that the judges memorize the real meaning of each point on the scale.

TEMPORARY ASPECT

This indicates the order of appearance of the attributes. Judges can also detect the order in which certain attributes are manifested. This order of appearance for physical properties (oral texture) is determined by the way in which the product is treated. Therefore, by controlling the application of forces in a certain way, certain attributes are exclusively manifested.

However, in certain properties (aroma and flavor), chemical composition of the sample and certain physical properties can alter the order in which certain attributes are detected.

INTEGRATING ASPECT

This tells us what the overall impression of the product is. There are several ways to integrate all the sensory properties of the product under study:

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- Total intensity of aroma or flavor: It measures the global impact of the aromatic components of a product.
- Mixture / Amplitude: Well-trained judges are sometimes able to assess whether aroma or flavor components are balanced (fit together). On certain occasions, the products do not have to be balanced if a certain note is to prevail over the others.
- Global difference: On certain occasions, important decision-making depends on the relative differences between a given product and a control or standard. Descriptive information provides an exact idea of the font and size of the differences.
- Hedonic evaluations: Training changes personal preferences of the judges. In knowing the various attributes of a product, judges tend to place a different weight on the attributes than they would valuate as consumers. Therefore, a hedonic judgment should not be requested from judges involved in descriptive analysis.

TYPE OF SCALES

Sensory analysis is an instrument or tool to record a panelist's reaction to any property that can be converted into numbers. For this reason, there are different types of scale:

- Response scale.
- Measurement scale.
- Ordinal scale.
- Interval scale.
- Proportion scales.

MEASUREMENT SCALE

Relationship between a property and the numbers used to represent the values of that property (for example, numbers recorded by the panelists).

ORDINAL SCALE

Scale in which the order of the assigned values corresponds to the order of intensities perceived for the property that is being evaluated.

INTERVAL SCALE

Scale that, in addition to having the attributes of an ordinal scale, is distinguished by the fact that equal differences between numerical values correspond to equal differences between perceived intensities.

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TYPES OF TESTS

These tests are used to qualitatively and quantitatively characterize one or more sensory attributes and can be performed on one or more samples. These tests are classified into:

- Simple descriptive tests.
- Quantitative descriptive analysis (QDA) and sensory profile.

SIMPLE DESCRIPTIVE TESTS

These tests allow obtaining a qualitative description of the particular properties that contribute to the overall characterization of the sample. This test is useful for training judges or fine-tuning preliminary vocabulary for more complex descriptive techniques.

Application: These tests are applied to:

- Identify and describe the properties of one or more samples.
- Stablish the sequence in which these properties are perceived.

Participants: It is made up of a group of tasters. A description of the sensory properties (qualitative study) and their measurement (quantitative study) are made. The tasters are trained during six or eight sessions to determine attributes that characterize the perceived sensations. About ten people are employed per evaluation.

Procedure for carrying out the descriptive test: The test can be carried out with one or more samples. When evaluating more than one sample per session, the order of service is of great importance. A random order of samples is recommended for each panelist.

Interpretation of the results: The results are gathered and based on the frequency with which each descriptive term has been used. A list of these results applicable to the analyzed sample is drawn up. It is often very helpful to have an open discussion at the end of the test.

QUANTITATIVE DESCRIPTIVE ANALYSIS (QDA) AND SENSORY PROFILE

They are tests or theoretical evaluation methods that allow to determine, in a reproducible way, sensory properties of a product using a list of terms (lexicon) previously established by means of simple descriptive tests. The different properties that contribute to forming the overall sensory impression of the sample are scored with an intensity scale. Results are used to establish a sensory profile of the product.

Application: These descriptive tests are recommended for:

- Development of new products.
- Establish the nature of the differences between products.
- Quality control.
- Provide sensory data to correlate with consumer and instrumental data.

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Participants: A group of selected panelists or experts, specially trained for this method, is required. Depending on the case, the panel leader can use it to lead the training and to control the discussion and establish consensus.

Procedure for carrying out the descriptive test: Preliminary tests (or training) are carried out with the range of products to be evaluated, in order to determine which organoleptic properties are important to characterize and differentiate them. The results of these tests are used to establish a lexicon of descriptive terms and the experimental procedure that will then be used to analyze the samples. The panel is trained with the methodology developed and particularly in the use of the lexicon. At this stage, it is useful to have a set of reference products, pure compounds or natural products that have a particular smell, taste, texture or visual (appearance) properties (Table 1). In the tasting sessions, panelists evaluate the samples based on the lexicon, scoring each property present with an intensity scale.

Table 1. Example for lexicon used for the descriptive analysis of *Fondillón*.

Attributes	Definition	References and intensities
Flavor		
Alcohol	A flavor reminiscent of alcoholic compounds	Ethanol solution 7% = 2.0; ethanol solution 11% = 5.0; ethanol solution 18% = 9.5
Fruity	A flavor blend that is sweet and reminiscent of a variety of fruits	Citral 16 $\mu g \cdot L^{-1}$ = 6.0; isoamil acetate 30 $\mu g \cdot L^{-1}$ = 6.0; benzaldehyde 100 $\mu g \cdot L^{-1}$ = 6.0
Floral	A sweet, heavy aromatic blend of a combination of flowers	Geraniol 10 $\mu g \cdot L^{-1}$ = 6.0; β-ionona 0.10 $\mu g \cdot L^{-1}$ = 6.0
Vegetable	Flavor reminiscent of a variety of different vegetables	2-Isobutyl-3-methoxypyrazine 0.02 $\mu g \cdot L^{-1}$ = 6.0; *cis*-3-hexen-1-ol 70 $\mu g \cdot L^{-1}$ = 6.0; 1-octen-3-ol 1 $\mu g \cdot L^{-1}$ = 6.0
Spicy	Flavor reminiscent of different species, which are directly related to the passage of wine barrels	Eugenol 15 $\mu g \cdot L^{-1}$ = 6.0; anethole 70 $\mu g \cdot L^{-1}$ = 6.0
Animal	Flavor reminiscent of animals or products derivatives thereof	Albona butter flavor 6 $\mu g \cdot L^{-1}$ = 6.0; "le nez du vin" flavor no. 45 = 9.0
Toasted	Aromas reminiscent of roasted products and generally coming from the toasting of the barrels	Vainillin 20 $\mu g \cdot L^{-1}$ = 6.0; 2-acetylthiazole 5 $\mu g \cdot L^{-1}$ = 6.0
Sweet	The fundamental taste factor associated with a sucrose solution	Sucrose solution 4% = 2.5; sucrose solution 8% = 5.0; sucrose solution 16% = 9.5
Sour	The taste stimulated by acids, such as citric and malic.	Tartaric acid solution 0.05% = 2.5; tartaric acid solution 0.08% = 4.0; tartaric acid solution 0.20% = 9.5
Bitter	The taste stimulated by substances such as quinine or caffeine	Caffeine solution 0.05% = 2.5; caffeine solution 0.08% = 4.0; caffeine solution 0.20% = 9.5
Astringent	The complex of drying, puckering, and shrinking sensations in the oral cavity	Alum solution 0.05% = 1.5; alum solution 0.10% = 3.0; alum solution 0.20% = 6.0
Global		
Imbalance	Wine attribute or attributes that prevail over the rest, breaking the balance	Sour: tartaric acid 2 $g \cdot L^{-1}$ = 6; astringent: tannin 4 $g \cdot L^{-1}$ = 6; bitter: quinine sulphate 0.03 $g \cdot L^{-1}$ = 6; alcohol: ethanol 60 $mL \cdot L^{-1}$ = 6
Persistence	Time it remains in the mouth, the characteristic flavor of the fruit after swallowing the sample	5–8 s = 5.0; 15–18 s = 10
Visual		
Limpidity	Without particles or coloidal elements in suspension	Isolated elements = 5; without particles = 10
Color	Visual evaluation of the color intensity of the sample	Pantone 1675C = 2.0; pantone 201C = 4.0; pantone 200C = 6.0
Color int.	Depth of color when you put a text under the glass	If you can read the text = 1.0; if you can see the text but you can't read it = 5.0; if you can't see the text = 10

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Interpretation of the results: There are two fundamental ways to handle the data:

- The group leader tabulates the results and establishes a discussion to resolve the differences immediately after the panelists have finished the analysis. Based on the results of this discussion and if necessary, after a new analysis of the samples the group reaches a joint decision on the profile.
- The profile is obtained from the mean values of the scores assigned by each of the panelists to each descriptor.

APPEARANCE

- COLOR
  - Description.
  - Intensity.
  - Brightness.
  - Homogeneity.
- TEXTURE / CONSISTENCY
  - *Thickness:* Density of the product.
  - *Roughness:* Number of irregularities that can be seen on the surface of the product.
  - *Smoothness:* Absence of particles on the surface.
  - *Particle Interaction:* Amount of adhesion between particles or the amount of agglomeration of small particles.
- SIZE / SHAPE
  - *Size:* Relative size of the pieces or particles in the sample.
  - *Shape:* Description of the predominant shape of the particles: flat, round, spherical, square, etc.
  - *Distribution:* Degree of uniformity of the particles in the sample.
- SURFACE BRIGHTNESS: Amount of light reflected from the surface of the product.

TASTE: The flavor is the combination of:

- Aromatic families.
- Basic flavors.
- Somatosensory sensations.

- AROMATICS: The list of aromas contains more than a thousand words. The flavor families for baked goods are shown below as an example: aromatic granules, terms related to grain and dairy terms.

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❖ BASIC FLAVORS

- *Sweet:* The flavor stimulated by sucrose and other sugars, such as fructose, glucose, etc., and by other sweet substances such as saccharin, aspartame, and acesulfame potassium.
- *Acid:* The flavor stimulated by acids, such as citric, phosphoric, tartaric etc.
- *Salty:* The flavor is stimulated by sodium salts, such as sodium chloride and sodium glutamate, and in part by other salts, such as potassium chloride.
- *Bitter:* The taste stimulated by substances like quinine, caffeine and bitter hops.
- *Umami:* The taste stimulated by monosodium glutamate.

❖ SOMATOSENSORY SENSATIONS

- *Astringency:* The shrinkage or drying of the surface of the tongue caused by substances such as tannins or alum.
- *Heat:* The burning sensation in the mouth caused by certain substances such as capsaicin from red, black peppers.
- *Freshness:* The cold sensation in the mouth or nose produced by substances such as menthol and mints.

SEMISOLID ORAL TEXTURE

❖ FIRST COMPRESSION

- *Viscosity:* Amount of the product that slides off the tongue.
- *Firmness:* Force required to compress food between the tongue and the palate.
- *Cohesiveness:* Degree of deformation of a product before breaking. It is related to brittleness, chewiness and rubberiness.

❖ HANDLING

- *Number of Particles:* Number of particles in the mouth (relative number).
- *Particle size:* Size of the food particles.

❖ POST-TASTE

- *Mouth lining:* Amount of film that remains on the surfaces of the mouth.

SOLID ORAL TEXTURE

❖ SURFACE TEXTURE

- *Geometric:* Total amount of small and large particles on the surface.
- *Moisture / Dryness:* The amount of moisture or grease on the surface.

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❖ PARTIAL COMPRESSION

- *Elasticity:* Degree to which the sample returns to its original shape after a certain period of time.

❖ FIRST BITE

- *Hardness:* Force required to bite.
- *Cohesiveness:* Amount of sample that deforms rather than breaks.
- *Fracturability:* The force with which the sample breaks.
- *Bite uniformity:* Uniformity of force during the bite.
- *Moisture Release:* Amount of moisture / juiciness released from the sample.

❖ FIRST CHEWING

- *Hardness:* Force required to chew.
- *Cohesiveness:* Amount of sample that deforms rather than breaks.
- *Fracturability:* The force with which the sample breaks.
- *Adhesiveness:* Force required to re-extract the sample from the molars.
- *Density:* compactness of the cross section.
- *Crunchiness:* The noise and force with which the sample breaks or fractures.
- *Moisture Release:* Amount of moisture / juiciness released from the sample.

❖ DURING CHEWING

- *Moisture absorption:* Amount of saliva absorbed by the product.
- *Mass Cohesiveness:* Degree to which the sample is held together in a mass.
- *Mass adhesiveness:* Degree to which the sample adheres to the palate or teeth.

❖ RESIDUAL

- *Geometric:* Number of particles that remain in the mouth.
- *Fatty coating:* Amount of oil that remains on the surface of the mouth.
- *Adhesive Mouth Coating:* Adhesion / stickiness of the coating when striking the tongue on the palate.
- *Tooth packing:* Amount of product remaining in the tooth crevices.

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STEPS FOR THE FORMATION OF A TRAINED PANEL

The procedure for forming a sensory analysis panel is made up of 3 phases:

- Selection.
- Training.
- Validation.

❖ SELECTION

The objective of carrying out the selection phase of the is to recruit / incorporate as many candidates as possible who want to be part of the panel. By carrying out different tests, those whose aptitudes and attitudes represent the best qualities for the correct operation of the panel are chosen.

This recruitment can be carried out within the same company (through the selection of internal personnel, avoiding selecting those who are experts in the product to be analyzed) or externally, through a call published in the media, the company's consumer database, or people related to the business.

The tests used for the selection of sensory judges have the objective of knowing their capacity against sensory stimuli and familiarizing them with the methodology and materials used in sensory analysis.

These are some of the tests that are carried out based on the type of information that needs to be collected to ensure that judges have the appropriate training to be selected:

- *View:* Testing is necessary to ensure that candidates do not have abnormal vision, that they are unable to discern between colors. For this purpose, for example, the Ishihara test can be used to detect people who suffer from some degree of color blindness. This test consists of a series of figures in which numbers can be distinguished within. A person with normal vision is able to differentiate the numbers from the rest of the figure, while a person with a certain degree of color blindness is unable to do so (Figure 1).

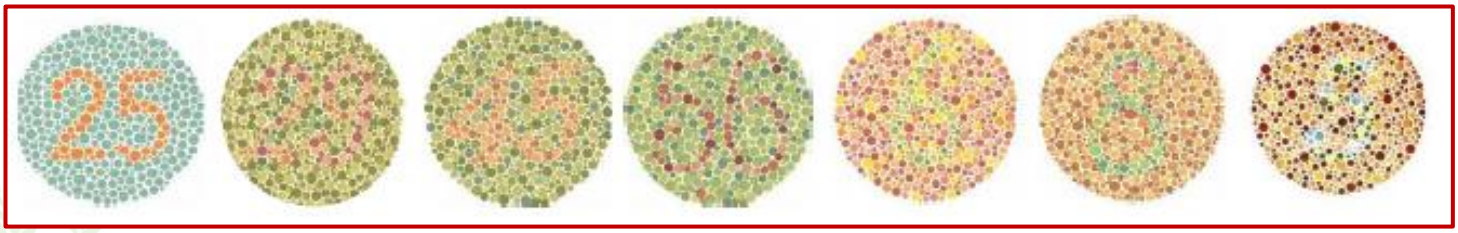

Fig. 1. Example of some figures belonging to the Ishihara test.

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- *Ageusias and anosmias:* Candidate judges need to be tested for their sensitivity to substances that may be present in small amounts in products. An essential test in this type of selection is the recognition of basic flavors and aromas typical of everyday life (Figure 2). To do this, concentrations of sweet, salty, sour, bitter and umami, and solutions with aromas easily related to typical products will be prepared to know if the judges are able to recognize them.

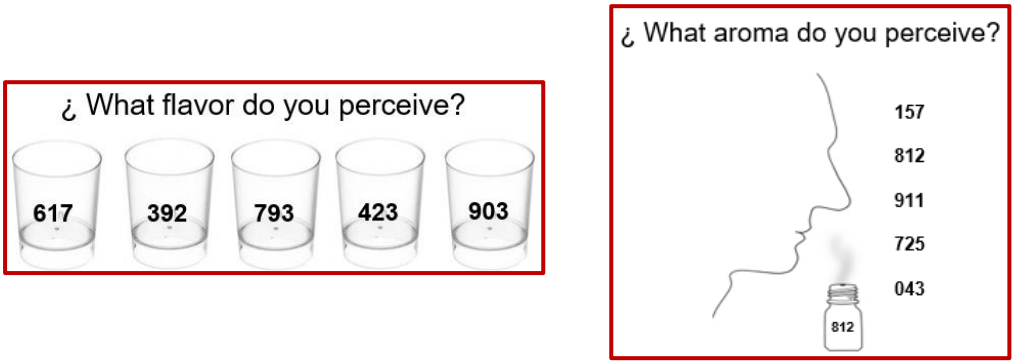

Fig. 2. Examples of taste and aroma tests.

- *Descriptive tests:* In order to test the ability of candidates to describe the sensations they perceive when testing a sample, it is necessary to carry out tests that allow them to express their sensations freely. This type of test is recommended for olfactory and texture stimuli. The judges are asked to describe typical products of everyday life based on previous experience with the product (for example, describe the texture of a boiled egg, a raw carrot, a maria cookie, etc.).

- *Working with scales:* Throughout the operation of the rear panel, it will be an essential requirement to be familiar with the working scales (linear, structured, etc.). Therefore, it is necessary to know if a judge is capable of working with these or not. A simple test may consist of the use of different scales for each of the tests to which the judge is submitted, to detect if any of these is an impediment (Figure 3).

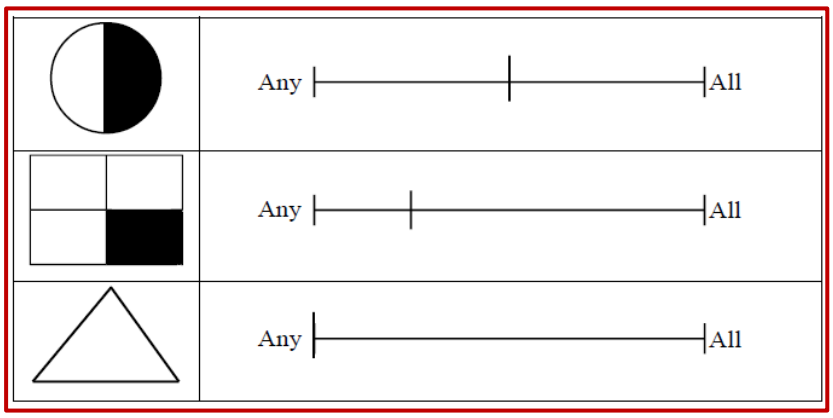

Fig. 3. Example of linear scales.

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❖ **TRAINING**

The training of a sensory analysis panel consists of providing the judges with the elementary principles of the techniques used and developing their aptitude to recognize and differentiate between the different sensory stimuli.

It is necessary that this entire phase takes place in the optimal working conditions (tasting room, light, temperature, absence of external odors, etc.) since, from this moment, these conditions will be a condition to be able to carry out any analysis, so the judges must already be used to them.

It is recommended that the sensory judges know the products they are going to test in great detail, so it is necessary to spend part of the training time in describing the production process, as well as the virtues and defects of the product.

The judges must be very well aware of the correct way to proceed when carrying out any sensory test: what they should describe and what not, how to fill in the questionnaire, serving temperature, number of chews, whether to swallow or not , etc.

Depending on the type of product to be evaluated, the training sessions will be distributed giving priority to the key attributes that allow characterizing the product. For example, if it is a panel that will be in charge of analyzing wine, the sense of smell and taste will be prioritized throughout the training; In the case of a panel trained to analyze cheese, much of the time will need to be spent training textural attributes and characteristics.

It is highly recommended not to leave aside any of the tests indicated below when training a sensory analysis panel:

- *Use of scales:* They must know perfectly all the scales with which the panel will work, being able to order any stimulus under study based on its concentration.
- *Descriptors:* They are one of the main focuses of attention when carrying out a sensory analysis panel training. These will allow a product to be evaluated following the scientific method, that is, it will allow the results to be reproducible and verifiable, by associating descriptors with reference products.
- *Flavor and odor detection and recognition:* Discriminatory techniques are used so that the judges learn to work with very low concentrations of a stimulus, as well as so that they learn to avoid complementary stimuli to focus on the stimulus under study.
- *Detection and recognition of defects:* Extensive work is being done on the identification and quantification of those defects that may appear in the products under study. It must be ensured that any product that later passes through the sensory analysis panel and presents a defect, whatever it may be, must be identified.

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❖ **VALIDATION**

The validation of the results is one of the most important stages in the formation of a sensory analysis panel, since it allows to demonstrate the correct functioning of this as a whole. This stage is carried out at the end of the training and must be periodic during the operation of the training, to demonstrate the effectiveness and good behavior of the panelists.

There are two concepts that must be considered when validating the results of a panel: repeatability and reproducibility. The first of them refers to the effectiveness of the panel or a judge to repeat evaluation results during the same evaluation session, while reproducibility studies this effectiveness, but in different sessions. For this, it is recommended, for example, the use of repeated samples in the same tasting session and repetitions between sessions. In this way, the deviations of the panel as a whole and of each of the judges can be calculated individually.

INTERPRETATION OF THE RESULTS:

The results obtained are interpreted by measuring the deviations using different statistical mechanisms. The simplest to use, and therefore the most widely used, are error and analysis of variance.

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EXAMPLE OF INFORMATION REPORTED IN RESEARCH ARTICLES:

As an example of how all this information must be summarized in a research article, here is the section "Descriptive Sensory Analysis with Trained Panel" of the article entitled "Volatile composition, sensory profile, and consumers' acceptance of *Fondillón* (Issa-Issa et al., 2019).

"Fifteen panelists (5 women and 10 men) aged 24–61 years (mean age 38 years) participated in this study which took place at the facilities of the Regulatory Board of the Alicante Protected Designation of Origin, Alicante PDO, in Alicante (Spain). They were (i) selected (3 sessions of 1.5 h), according to their results in previous sensory discrimination, ranking, and recognition tests, (ii) trained (12 sessions of 1.5 h, during4 months) (they were fully trained in descriptive sensory of wines from the Alicante PDO), and (iii) validated (2 sessions of 1.5 h), and are included in the control tools of the Regulatory Board to control the quality of their wines; this tool is included among those certified by the ISO/IEC 17065 : 2012, with the reference number 118/C-PR198. These panelists are paid for their involvement in the current study and any other evaluations they perform. No orientation session was needed because the panelists of the Alicante PDO are used to evaluate this type of wine. During the panel training, the panelists were instructed about the tasting protocol, the questionnaire structure and the order of the attributes to be evaluated, the lexicon(Table 1), and the scale to be used"

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36. Determination of glycoalkaloids (α – solanine and α – chaconine)

EQUIPMENT AND REAGENTS:

Equipment and reagents	
Equipment	Description
Ultrasound bath	With timer and adjustable temperature
Solid Phase Extraction chamber	Preferably with 12 SPE columns slots. Equipped with vacuum pump.
HPLC	Shimadzu Prominence –i LC – 2030C Plus
Column	Supelcosil LC-18, 25 cm x 4,6 mm, 5 µm
Pre-column	Supelcosil LC-18 Supelguard Cartridge, 2 cm x 4 mm, 5 µm
Automatic pipettes	Volumes : 1 – 10 µl, 20 – 200 µl, 100 – 1000 µl, 1 – 10 ml
Laboratory Rotary Evaporator	Buchi Rotavapor R-100
Reagent and Materials	
Ultrapure deionized water	Milli-QR system
Disposable gloves	
Syringe filter	0,22 µm
SPE cartridges	Reversed Phase
Methanol	HPLC grade
Acetonitrile	HPLC grade
KH_2PO_4	pure for analysis
α – solanine	phyproof Reference Substance
α - chakonine	phyproof Reference Substance

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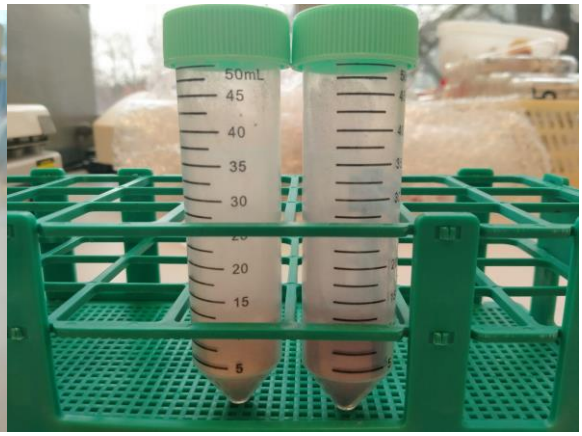
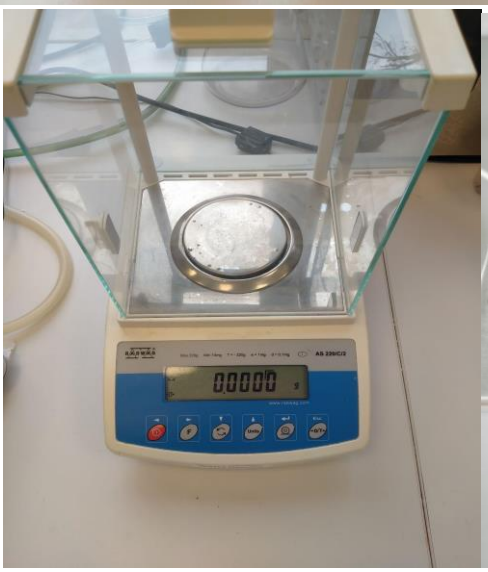
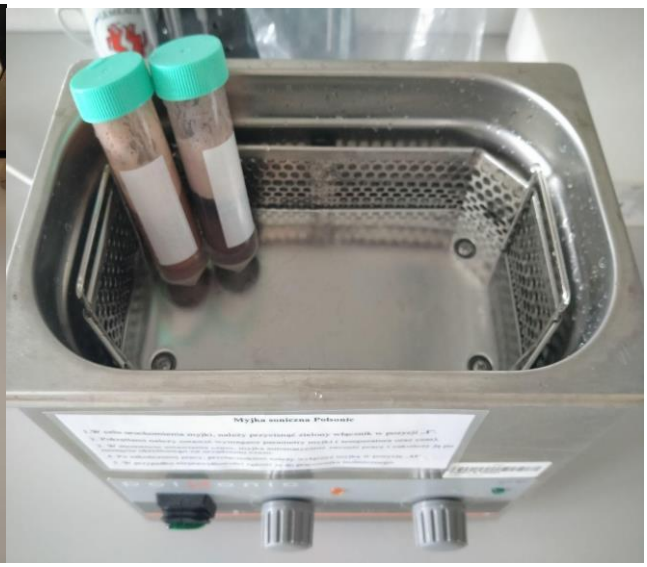
METHOD DESCRIPTION:

Extraction of potato glycoalkaloids

5 grams of dried potato product is weighted into a plastic 50 mL centrifuge tube, treated with 25 mL of methanol and placed in ultrasound bath for 30 minutes at room temperature.







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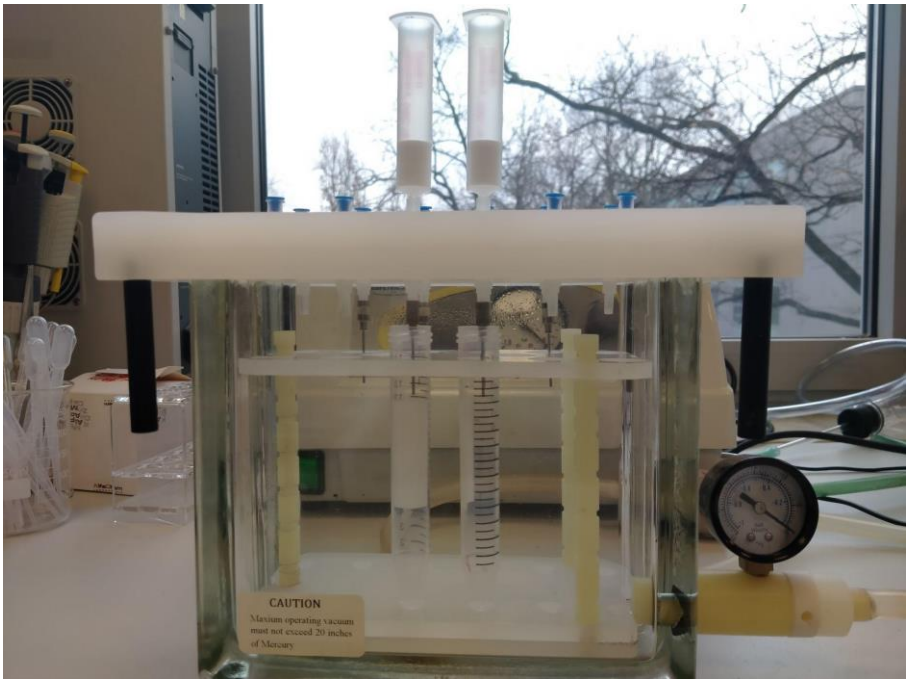
The mixture was filtered through filter paper into a volumetric flask and filled with methanol to 50 mL. SPE procedure starts with conditioning the SPE cartridge with 10 mL of methanol and 10 mL of water in that order.



Then 5 mL of potato product extract is mixed with 8 mL of water and is applied to the SPE cartridge. Sample is cleaned by using 5 mL of 40% methanol and then 15 mL of pure methanol is used to rinse the analytes from the solid phase. Collected extract is placed in a 50 mL round bottom flask and evaporated to dryness on a rotary evaporator. Dry residue is dissolved in 1 mL of methanol and filtered through a 0,22 µm PTFE syringe filter.

External calibration

External calibration was accomplished using standards of 1 – 100 µg L^{-1} of each of the two analytes (α – solanine and α – chaconine). Analysis was run in triplicate.

High-performance liquid chromatography (HPLC) conditions

Analysis are performed on Shimadzu Prominence - LC – 2030C Plus high performance liquid chromatograph with UV detection. Column used is Supelcosil LC-18 (25 cm x 4,6 mm, 5 µm) and Supelcosil LC-18 Supelguard (2 cm x 4 mm, 5 µm) as a pre-column. Mobile phase consists of 30% acetonitrile and 70% 0,1M KH_2PO_4 with isocratic flow of 1 mL/min. UV wavelength is 200 nm, injection volume is 20 µl and temperature is set to 70 ˚C. Analysis is carried out in 10 minutes and elution order is α – solanine first and then α – chaconine.

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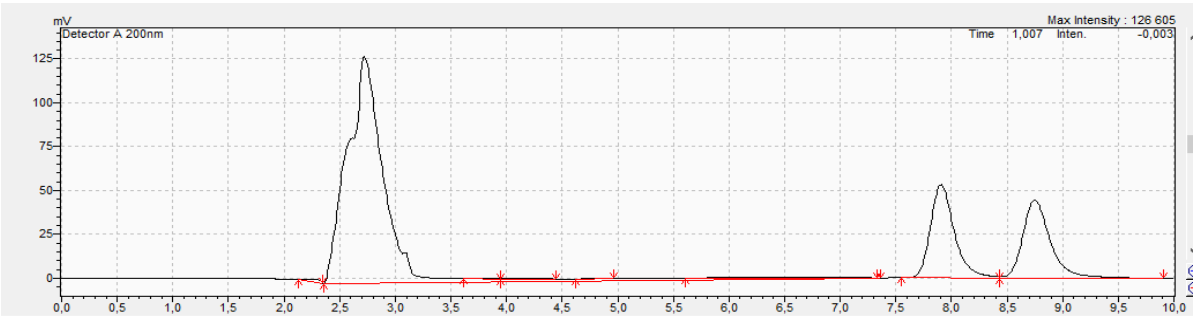
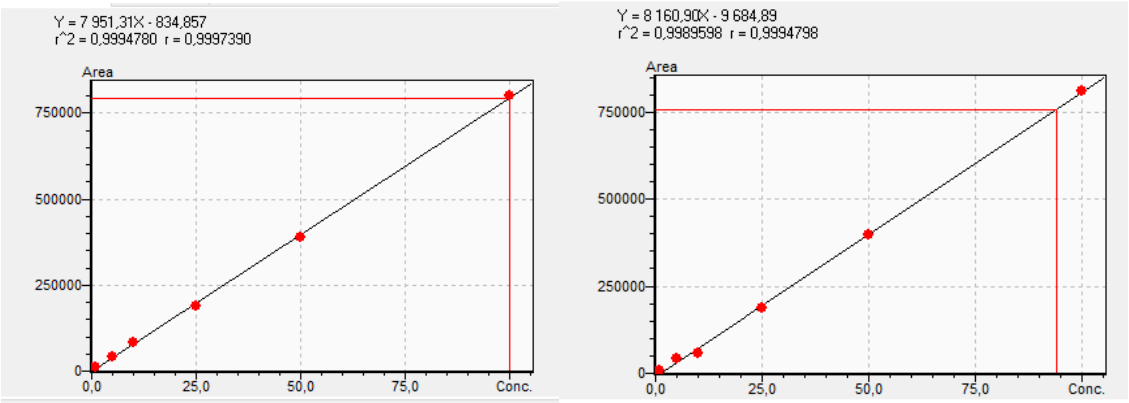
INTERPRETATION OF THE RESULTS:

The following figure represents chromatogram obtained from measurements of sample containing 100 µg L^{-1} of α – solanine (retention time of 7.9 minutes) and α – chaconine (retention time of 8.5 minutes):



For each concentration level (1, 5, 10, 25, 50, 100 µg L^{-1}) of each analyte - mean peak area was calculated and used to create calibration curve presented below (Left - alpha solanine calibration curve; right - alpha chaconine calibration curve).



Recommendations:

Make sure that all glassware are as clean as possible and reagents used are of HPLC purity. Each analysis should be conducted in at least 3 repetitions.

LITERATURE:

Koichi Saito, Masakazu Horie, Youji Hoshino, Norihide Nose - "High-performance liquid chromatographic determination of glycoalkaloids in potato products:, Journal of Chromatography, 508 (1990) 141-147

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37. Determination of Polycyclic aromatic hydrocarbons (PAHs) in grilled beef meat

EQUIPMENT AND REAGENTS:

Equipment	
Equipment	Description
Milly Q-System	
HPLC-FLD	HPLC unit (Jasco, Japan) equipped with PU-1580 HPLC pump, an AS-950 auto sampler with a 20-µL loop and a FP-920 fluorescence detector. Column C18 Supelcosil LC-PAH (25 cm length; 4.6 mm internal diameter, 5-µm particle size) (Supelco, Bellefonte, PA, USA).
Centrifuge	
Disposable charcoal barbecues	
Digital thermocouple with a surface probe	
Reagent and Materials	
Standard mixture (PAH Calibration Mix)	10 µg/mL of naphthalene, naphthalene, acenaphthene (Ac), acenaphthylene, fluorene (F), phenanthrene (Pa), anthracene (A), fluoranthene (Fl), pyrene (P), benzo(a)anthracene (BaA), chrysene (Ch), benzo(b)fluoranthene (BbF), benzo(k)fluoranthene (BkF), benzo(a)pyrene (BaP), indeno(1,2,3-cd)pyrene (IP), benzo(ghi)perylene (BgP), dibenzo(ah)antracene (DhA) Supelco (47940-U, Bellefonte, PA, USA).
Acetonitrile HPLC Grade	
Internal standard, Tryphenylene	(purity ≥ 98%)
Acetone HPLC Grade	
Hexane HPLC Grade	
Water HPLC plus	
Methanol	
Ethyl acetate	
Anhydrous magnesium sulfate	
Sodium chloride	
Amber flask	
Centrifuge tube	

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METHOD DESCRIPTION:

The overall method consists in three steps:

Preparation of samples

Beef meat samples (5 replications) are cooked in disposable charcoal barbecues and the fire is set according manufacture's instructions. The grilling temperature is maintained around 200 °C with an internal temperature of 75°C measured by a digital thermocouple with a surface probe. Samples are turned once during grilling at half of the total cooking time (10 min). After cooking, all samples are frozen until PAH analysis.

PAHs Extraction

PAHs extraction is performed using a acetonitrile-based extraction according to the method descibed by Silva et al. (2018). In a 50-mL centrifuge tube, grilled meat sample (5 g) is mixed with the spiking standard and/or internal standard solutions and 5 mL of deionized water and vortexed for 1 min, then 5 mL of acetonitrile is added and shacked vigorously by hand. Afterwards, anhydrous magnesium sulphate (2.5 g) and sodium chloride (2.5 g) is added, followed by shaking and centrifugation at 4000 rpm for 5 min. Subsequently, 2 mL of the upper layer acetonitrile extract is evaporated to dryness under nitrogen stream at room temperature. The residue is then dissolved in 100 µL of acetonitrile and injected into the HPLC-FLD.

Quantification PAHs in HPLC-FLD

Following PAHs extraction, the sample is injected into a HPLC unit equipped with a column C18 Supelcosil LC-PAH (25 cm length; 4.6 mm internal diameter, 5-µm particle size) thermostated at 32 °C. Three solvents are used for mobile phase with a flow rate 1 ml/min: 75% methanol in water (A), methanol (B) and ethyl acetate (C). The linear gradient program is: 0–18 min, 0–80% B in A; 18– 19 min, 80–100% B in A; 19–20 min, 100–90% B in C; 20–28,5 min, 90–82% B in C; 28,5–37,5 min, 82–80% B in C; 37,5–40 min, 80–100% B in C, 40–45 min 100– 0% B in A, rinsing and re-equilibration of column to the initial conditions. Excitation/emission wavelengths selected are 276/330 nm for Na, Ac and F; 250/ 336 nm for Pa; 250/402 nm for A; 270/460 nm for Fl; 270/390 nm for P, BaA and Ch; 260/430 nm for BbF; 290/410 nm for BkF, BaP, DhA, and BgP.

INTERPRETATION OF THE RESULTS:

The PAHs identification is based on the retention time of and on co-elution with standards. A calibration curve is prepared, using blank samples spiked with appropriate amounts of PAH calibration mix and internal standard (40 ng/g). Calibration curves with seven concentration levels is prepared in the interval between 0.2 and 30 ng/g for PAH8, whereas for other PAHs 9 concentration levels are prepared in the interval 0.2 and 60 ng/g. Limits of detection (LOD) and limits of quantification (LOQ) is calculated based on the calibration curve parameters. Recovery

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studies were carried out to determine the accuracy of the extraction procedure; for this purpose, different types of grilled and smoked samples were analyzed with and without spike at one level of 4 ng/g depending on PAHs concentration in unspiked samples. Recovery studies is carried out to determine the accuracy of the extraction procedure; for this purpose, different types of grilled and smoked samples are analyzed with and without spike at different levels depending on PAHs concentration in unspiked samples.

DEVICE PERFORMANCE

The analytical performance of the extraction method for reliable chromatographic quantification of PAHs is evaluated. The method was validated in accordance with the guidelines established by ICH (International Conference on Harmonization (2005) recommendations for the 14 PAHs under study.

LITERATURE:

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38. Determination of sodium chloride in cheese and butter

EQUIPMENT AND REAGENTS:

Equipment	Description
Analytical balance	with 0,0001 g resolution
Cheese grater	
Blender	
potentiometer	a potentiometer with a millivolt (mV) scale capable of a 1 mV precision or better, e.g. a Mettler Toledo FE20 FiveEasy™ pH Meter with Thermo Scientific's Orion Half-Cell Silver/Sulfide Selective Ion Electrode and a Thermo Scientific Orion Double Junction Reference Electrode
Magnetic stirrer plate	
Volumetric flask	100 mL
Erlenmayer flasks	150 mL and 250 mL
Beakers	200 mL
Glass funnels and filter paper	
Glass pipettes	50 mL volumetric pipette, graduated pipettes: 20 mL and 10 mL
Buret	25 mL
Reagents and Materials	
Distilled water	
NaCl	to prepare the primary standard 0.1 M solution
$AgNO_3$	to prepare 0.1 M solution which must be accurately standardized with NaCl solution
HNO_3	1 M solution
NaOH	1 M solution
K_2CrO_4 or Na_2CrO_4	5% solution

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METHOD DESCRIPTION:

Sample preparation

Cottage cheese samples should be blended to form a homogenous pulp; hard cheese, after removing the outer layer, should be grated.

Cheese, potentiometric titration

Weigh accurately approximately 4 g of cheese (note the exact weight). Put it into a 100 mL volumetric flask, add 50 mL of warm distilled water (50 °C), then 10 mL of 1M NaOH solution, and stir the content until the cheese gets into the solution. After cooling to room temperature, add 20 mL of 1M HNO_3, and make up the volume with distilled water. After the solution is thoroughly mixed, separate the insoluble matter on a folded paper filter collecting the solution in a dry 200 ml beaker. The electrode should be rinsed with distilled water, and then dried with soft tissue. Transfer 50 mL of the examined solution with a pipette to a 150-mL Erlenmayer flask and put the electrode into the solution, note the potential value. Titrate with standardized c.a. 0.1 M $AgNO_3$ using 0.5 mL portions of the titrant and record the potential value after each addition. During the addition, the solution should be stirred by means of a magnetic stirrer. The titration should be finished when 25 mL of $AgNO_3$ solution has been added.
Draw the titration curve, read the end point and calculate the percentage of NaCl in cheese.

Butter (the Mohr titration method)

Weigh about 5 grams of butter in triplicate (note the exact weights) and put them into 250-mL Erlenmayer flasks, add 100 mL of boiling distilled water, and stir for 5 – 10 min. Add 2 mL of 5% sodium or potassium chromate indicator and titrate with standardized c.a. 0.1 M $AgNO_3$ to the first visible pale red-brown color that persists for 30 s (the end point). Record the volume (in ml) of titrant used. Calculate the chloride content and the sodium chloride content of each replicated sample.

INTERPRETATION OF THE RESULTS:

General remarks

The determination of chloride in cheese by **potentiometric titration** is based on the reaction between extracted chloride ion and silver(I)-ion (Ag^+) from silver nitrate ($AgNO_3$):

$$Ag^+_{(aq)} + Cl^-_{(aq)} \rightarrow AgCl_{(s)}$$

The course of titration is monitored potentiometrically by using a silver electrode as working and the calomel electrode as the reference.
During titration, the concentration of chloride ions in the solution decreases due to the formation of hardly dissolving silver chloride. As a result, the electrode potential gradually

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increases. After the whole chloride ion is titrated, the potential of the silver electrode rapidly increases. Further addition of the silver nitrate solution causes a slow increase in the potential of the electrode, as it is sensitive to the presence of silver (I) ions, the concentration of which slowly increases in the solution.

The end point (equivalence point of titration) is established from the titration curve (the potential between two electrodes as a function of additional titrant volume) with appropriate precision (see figure 1). It corresponds to the amount of silver nitrate [in ml] spent at the equivalence point of the titration.

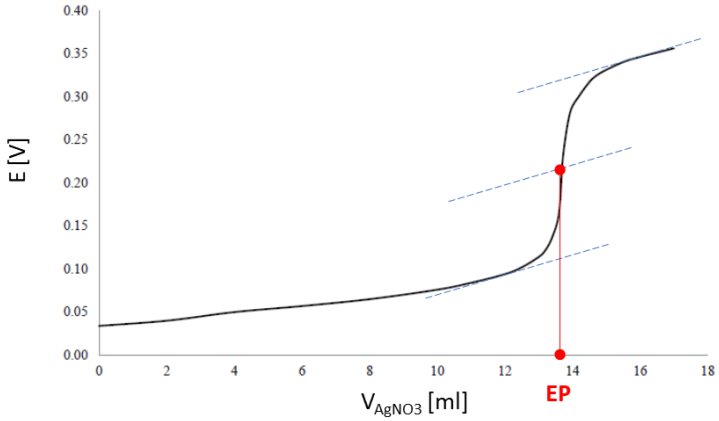

Figure 1. Potentiometric titration curves for chloride ion in cheese determination.

Mass share of sodium chloride (in %) in cheese samples is calculated in the following way:

$$\%NaCl = \frac{ml(EP)\ of\ AgNO_3 \times cAgNO_3 \times 2 \times M_{NaCl}}{g\ of\ sample} \times 100\%$$

The Mohr method consists in producing orange silver chromate during chloride titration with silver nitrate in the presence of potassium chromate. The orange-coloured solid of Ag_2CrO_4 appears after all chloride from the solution has been precipitated in the form of AgCl.

$$Ag^+_{(aq)} + Cl^-_{(aq)} \rightarrow AgCl_{(s)}$$

$$Ag^+_{(aq)} + CrO_4^{2-}{}_{(aq)} \rightarrow Ag_2CrO_4\ \text{(orange solid)}$$

The chloride and sodium chloride content in butter samples are calculated in the following way:

$$\%\ chloride = \frac{ml\ of\ AgNO_3 \times cAgNO_3 \times M_{Cl-}}{g\ of\ sample \times 1000} \times 100\%$$

$$\%\ NaCl = \frac{ml\ of\ AgNO_3 \times cAgNO_3 \times M_{NaCl}}{g\ of\ sample \times 1000} \times 100\%$$

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39. Determination of taurine and histidine dipeptides (carnosine, anserine and derivatives) content by HPLC

EQUIPMENT AND REAGENTS:

Equipment and reagents	
Equipment	Description
HPLC with fluorescence detector	HPLC 1100 Series system with the fluorescence detector (266-450 nm) Separations on a Zorbax Eclipse - AAA group (4.6 x 150 mm, 3.5 µm) (Agilent Technologies).
Homogenizer Mixer B-400 (Buchi)	
Centrifuge (Sigma)	10,000-12,000 x g, 15-30min
Spectrophotometr	
Optional equipment	
Equipment	Description
Freeze-dryer	24 h; Christ Alpha 1–4 LSC; Martin Christ GmbH, Germany
Automatic pipettes	Range from 2 µl to 5000 µl
Reagent and Materials	
methanol HPLC	
acetonitrile-HPLC	
OPA [o-phthaldialdehyde reagent]	
Phase A	40 mM Na_2HPO_4 (pH 7.8) in HPLC water
Phase B	Acetonitrile : methanol : water (45:45:10)
L-carnosine, L-anserine, taurine standards	Analytical standards (Merck)
Biuret reagent	sodium potassium tartrate, copper sulfate x 5 H_2O, potassium iodide, dissolved in 0.2 M NaOH
bovine serum albumin	For the standard curve preparation
SPE 0.20 mm membrane filters	Millex Samplicity Filter, Merck
Glass or plastic cuvettes	550 nm
Whatman #4 filter paper	

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METHOD DESCRIPTION:

The method consists of three main steps.

- Preparation of the sample: homogenization, centrifugation and derivatization with OPA,
- Protein analysis Biuret method
- Chromatographic separation and fluorescence detector detection

Sample preparation

Samples can be analyzed in fresh state or after freeze-drying. The powdered lyophilized or fresh samples are homogenized (Mixer B-400, Buchi, Switzerland) with precooled (4°C) redistilled iced water (1:1) using 2 cycles of 2 min each cycle. The homogenate is centrifuged at 12,000 × g for 30 min at 4°C (Sigma Ltd., Newtown, UK). The supernatant is then filtered through a Whatman #4 filter paper. The hydrophilic fractions of the sample is then deproteinized by filtration through glass wool, the addition of 3 vol of methanol (HPLC grade), keeping in the freezer for 30 min, and final purification by centrifugation (12,000× g) (Sigma, 3K30). Sampleas can be also deproteinized by an SPE system (Solid Phase Extraction).

Protein analysis

Protein content is determined using the biuret method. Basically, prepare standards from bovine serum albumin, preferably calibrated using absorbance at 280 nm and the extinction coefficient. Using 5 ml color reagent to 1 ml sample a recommended range is 0.5 to 20 mg protein. Prepare a reference tube with 1 ml buffer. If possible, dilute unknowns to an estimated 1 to 10 mg/ml with buffer; a range of dilutions should be used if the actual concentration cannot be estimated. Use 1 ml sample per assay tube. Add 9 ml Biuret reagent to each tube (9 g sodium potassium tartrate, 3 copper sulfate x 5 H_2O, 5 g potassium iodide, all dissolved in order in 400 ml 0.2 M NaOH before bringing to final volume of 1 l). Vortex immediately, and let stand 20 min. Read the absorbance at 550 nm.

Separation / Detection

The methodology of HPLC analysis is based on Henderson et al. (2000). The sample solution is reacted with O-phthalaldehyde (OPA pre-column derivation method) before being injected into HPLC. Chromatographic analysis is performed in an HPLC 1100 Series system (Agilent Technologies) equipped with a fluorescent detector. The separation is monitored at a wavelength of 340/450 nm (excitation/emission) for 0–15 min and a wavelength of 266/305 nm after 15 min. The chromatographic separation is developed using a ZORBAX Eclipse-AAA column (4.6 × 150 mm, 3.5 µm, Agilent Technologies). Mobile phases consist of solvent A, containing 40 mM Na_2HPO_4, pH 7.8 in water and solvent B containing acetonitrile: methanol: water (45:45:10). The gradient is programmed as follows: 0% B for 1.9 min, linear gradient from 0% to 57% B for 18.1 min, up to 100% B at 18.6 min and stay at 100% B for four minutes,

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then up to 26 min at 0% B. L-carnosine, L-anserine (as L-anserine nitrate salt) and taurine standards from Sigma (Sigma-Aldrich Co., St. Louis, MO, USA) are used for the method validation.

INTERPRETATION OF THE RESULTS:

Characterization of the single component is carried out via the retention time. The PDA spectra are measured over the wavelength range of 200–500 nm in steps of 2 nm. The retention times are compared to those of the authentic standards.

DEVICE PERFORMANCE

The method is validated in accordance with the requirements for the set methods. L-carnosine and L-anserine (as L-anserine nitrate salt) and taurine from Sigma (Sigma-Aldrich Co., St. Louis, MO, USA) in series of dilutions are used for the method validation. The linear relationship between dipeptide and aminoacid standards of five different concentrations and peak areas are usually found. Reproducibility of the method (92–104%) is verified after the addition of the standard dipeptides to analyzed sample homogenate extracts.

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40. Evaluation of trapping devices in field cages for monitoring fruit fly populations

EQUIPMENT AND REAGENTS:

Equipment	
Equipment	Description
Data logger	Recording of temperature and relative humidity
Materials	
Cage(s)	Square (2x2x2 m) plastic-screen cage(s). For each cage: 24 m^2 of fine mesh net
Fruit tree(s)	1.50/1.90 m of hight and 50-90 cm of canopy diameter
Shading net	About 2x2 m
Trap(s)	Of the type(s) to be assessed
Attractant(s)	Of the type(s) to be assessed
Laboratory colony of fruit fly	Specifically, the species you want to use in the trials
Small cage(s)	Trasportation cage(s) (for example: plastic-screen cage 30x30x30 cm)
Granulated sugar	
Water	
Cotton pads	

METHOD DESCRIPTION:

The overall method consists of three steps:

Preparation of cages

In the center of each cage, a fruit tree is placed. Fruits, if any, are removed from the trees. Field cage is protected from direct sunlight by placing a shading net one meter above its top. The number of cages to be used depends on the number of traps to be assessed. One cage for each type of trap device.

Preparation of insects

One day before release in the field cages, 10-15 days-old adults are collected from the laboratory colony and placed into small cages in groups of 50 individuals (25 males and 25 females) with ad libitum access to granulated sugar and water (water-soaked cotton pads). The

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number of groups to be used depends on the number of cages used in the trial. One group (50 individuals) for each cage.

Release of insects

At test day, 50 adults (25 males and 25 females) are released into field cage. Thirty minutes later, the respective trapping devices, with the corresponding attractant, are placed in the field cages. A single trapping device is placed in each field cage on the tree at a height of 1.20-1.60 m. Flies have ad libitum access to granulated sugar and water (water-soaked cotton pads fastened to tree branches).

Experimental design

Trap captures (number of males and females entering the trap) are recorded at hourly intervals for 8 hours. Minimum 8 replicates must be run. Trapping devices must be rotated within field cages in clockwise manner per replication. The temperature and relative humidity are recorded with the help of a data logger placed in a field cage.

At the end of each test day, the cages are cleaned from insects and wiped to be ready for the next repetition.

INTERPRETATION OF THE RESULTS:

The Kolmogorov-Smirnov test is used to assess the normality of data distributions. Generalized Linear Models (GLMs) with Poisson distribution of error and a loglinear link function is used to determine the effects of treatment (trapping device), sex, temperature and their interactions on adult captures in each experimental day. The Bonferroni post-hoc test is carried out for multiple comparison at $\alpha = 0.05$.

If a male or female specific attractant is used, one-way ANOVA followed by the Tukey HSD test for multiple comparisons is used to determine the effect of treatment on the proportion of male or female captured.

LITERATURE:

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41. Extraction, identification and quantification of anthocyanins by LC-MS/MS: figs as an example

EQUIPMENT AND REAGENTS:

Equipment	
Equipment	**Description**
Micro pipettes	Range from 2 µl to 5 mL
Orbital bath	Unitronic 320 OR, Selecta, Barcelona, Spain
Ultrasonic bath	Model 3000512, Selecta, Barcelona, Spain
Centrifuge	Sigma 3−18 K; Sigma Laborzentrifugen, Osterode and Harz, Germany
Column	Mediterranea SEA 18 (10 mm L x 0.21 mm i.d., 2.2 µm), Teknokroma, Barcelona, Spain
High Performance Liquid Chromatography Mass Spectrometer	LCMS-8050 (APCI-8050, DUIS-8050), Shimadzu, Japan
Reagent and Materials	
MilliQ water	Millipore system
Methanol	LC-MS grade, Sigma-Aldrich, Steinheim, Germany
Formic acid (FA)	LC–MS grade (> 99 %), Fisher Scientific, France
Acetonitrile (ACN)	LC-MS grade, PanReac AppliChem, Spain
External standards	Delphin chloride; Cyanin chloride; Pelargodin chloride; Myrtillin chloride; Kuromanin chloride; Callistephin chloride; Pelargonidin-3-*O*-glucoside chloride; Keracyanin chloride; Cyanidin-3-*O*-arabinoside chloride; Pelargonidin-3-*O*-rutinoside chloride; Petunidin-6-*O*-glucoside chloride; Malvidin-3-*O*-galactoside chloride; Oenin chloride; Pelargonidin 3,5-di-glucoside
Chromatography vials	1.5 mL short thread vial, 32 x 11.6 mm, amber glass
Filter	Pore size 0.45 µm and diameter 25 mm nylon, Fiber lab, Barcelona, Spain
Threaded glass tube	10 mL

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MATERIALS AND METHODS:

1. Fruit materials

Figs (*Ficus ficaria* fruits) are given as an example of vegetable matrix for the extraction and identification of anhtocyanins in vegetables. For the determination of anthocyanins, the figs were collected in their optimum state of maturity and the analysis was carried out on the pulp and peel. First, a quick freeze with liquid nitrogen was carried out at -196°C and later the samples were frozen at -80°C until lyophilization. After this process, they were ground with an electric grinder and stored in vacuum packaging at -20°C. The samples were stored in the dark.

2. Extraction method

The extraction method condition is according to a published method (H.T. Hong et al., 2020) with some modifications. Consists of 0.5 g of finely ground freeze-dried figs powder shaking in a orbital bath with 4 mL cold extractant (methanol/water/formic acid (80:19.9:0.1, *v*/*v*)) for 10 minutes. Then it is sonicated by ultrasonic bath for 10 minutes. Next, the slurry is centrifuged at 4.000 rpm during 10 min. The supernatant is removed and the pellet residue re-extracted twice using the same procedure. Two milliliters of the supernatant is filtered through a 0.45 µm nylon Millipore membrane filter used for analysis. All extractions have to be carried out in triplicate.

3. Analytical method

The instrument used in this analysis is a LC-MS/MS 8050 High Performance Liquid Chromatography triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). The ISE source operates with a nebulizer gas flow of 3 L/min, drying gas flow of 10 L/min, a desolvation line (DL) temperature of 250°C and heat block temperature of 400°C. Selected ion monitoring (SIM) with a collision energy of -35V and full MS scans in positive mode between 100-1000 m/z. For the analysis was used a Mediterranea SEA18 (10 mm L x 0.21 mm i.d., 2.2 µm particle size) column maintained with a temperature at 50°C. The mobile phase A consisted of 0.1% formic acid (FA) in water (MilliQ) and the mobile phase B consisted of 0.1% formic acid (FA) in acetonitrile (ACN). The gradient elution is run using a flow rate of 0.400 mL/min as shown in Table 1. Injection volume sample was 10 µL.

Table 1. Gradient elution for anthocyanins determination.

Time (min)	A (%)	B (%)
0	95	5
2	95	5
10	5	95
11	5	95
12	95	5
16	95	5

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METHOD DEVELOPMENT:

14 anthocyanins (external standards) are needed for the optimization and subsequent quantification: Delphin chloride; Cyanin chloride; Pelargodin chloride; Myrtillin chloride; Kuromanin chloride; Callistephin chloride; Pelargonidin-3-*O*-glucoside chloride; Keracyanin chloride; Cyanidin-3-*O*-arabinoside chloride; Pelargonidin-3-*O*-rutinoside chloride; Petunidin-6-*O*-glucoside chloride; Malvidin-3-*O*-galactoside chloride; Oenin chloride; Pelargonidin 3,5-di-glucoside.

A concentration of 200 ppm is prepared for each anthocyanin. Three concentrations (0.1, 1 and 10 ppm) have to be obtained to perform a full scan in FIA (Flow Injection Analysis). Once it is verified that the full scan is carried out correctly, the optimization is performed with the MRM (Multiple Reaction Monitoring) method, which allows the acquisition of quantitative data with high sensitivity. The concentrations to be used are the same as for the full scan (0.1, 1 and 10 ppm).

Next, a table of the components is created for the qualitative and quantitative determination of the 14 anthocyanins. For example, the malvidin-3-*O*-galactoside chloride chromatogram is shown along with the calibration curve information (Figure 1 and Figure 2).

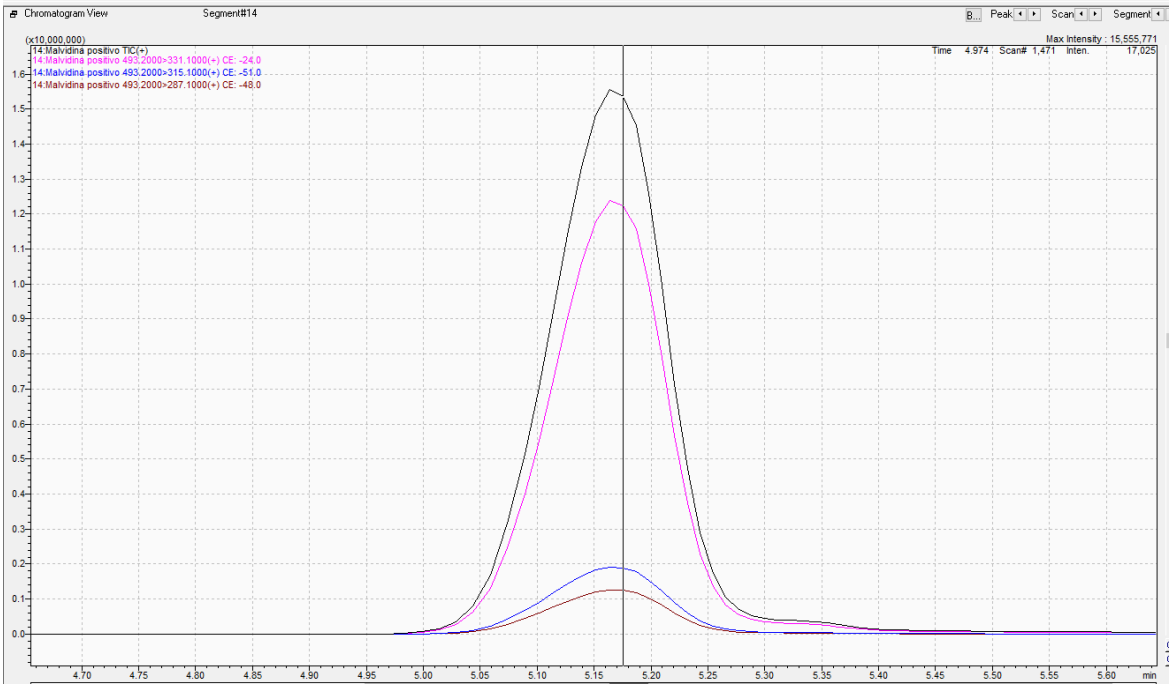

Fig. 1. Malvidin-3-*O*-galactoside chloride chromatogram.

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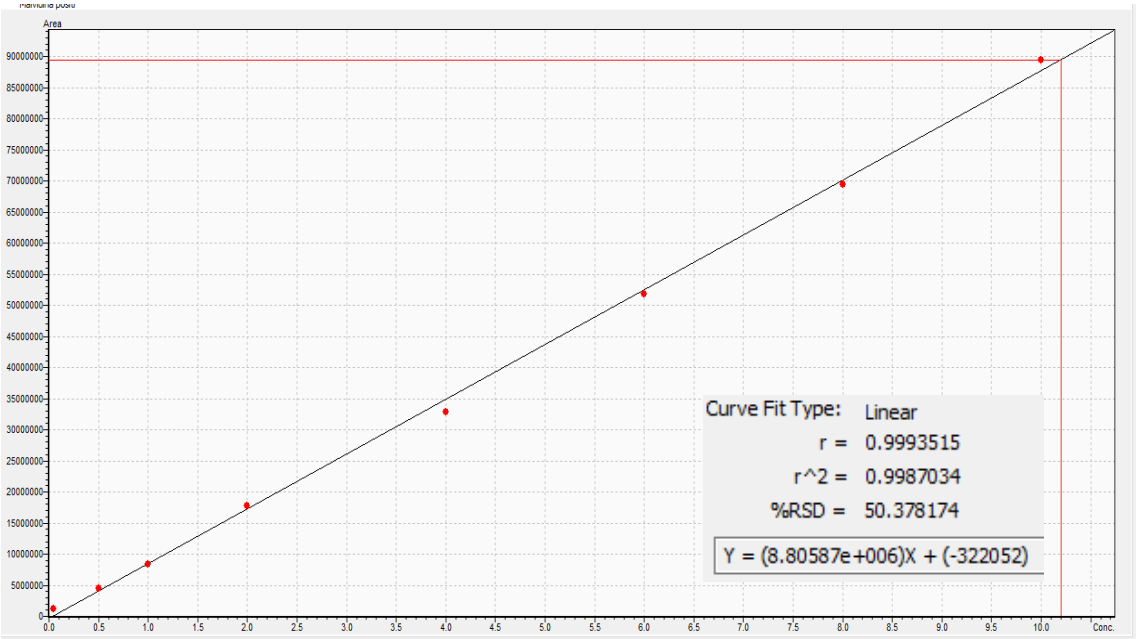

Fig. 2. Standard calibration curve of Malvidin-3-*O*-galactoside.

LITERATURE:

1. Hong, H. T., Netzel, M. E., O'Hare, T. J. 2020. Optimisation of extraction procedure and development of LC-DAD-MS methodology for anthocyanin analysis in anthocyanin-pigmented corn kernels. Food Chemistry, 319. doi.org/10.1016/j.foodchem.2020.126515
2. Hong H. T., Netzel, M. E., O'Hare, T. J. 2020. A dataset for anthocyanin analysis in purple-pericarp sweetcorn kernels by LC-DAD-MS. Data in Brief, 30. doi.org/10.1016/j.dib.2020.105495

Publications:

Caranqui-Aldaz, Jorge M., Raquel Muelas-Domingo, Francisca Hernández, and Rafael Martínez. 2022. "Chemical Composition and Polyphenol Compounds of Vaccinium floribundum Kunth (Ericaceae) from the Volcano Chimborazo Paramo (Ecuador)" Horticulturae 8, no. 10: 956. https://doi.org/10.3390/horticulturae8100956

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42. Food-Extract DNA Purification Kit Color Taq PCR Master Mix - Genetically Modified Organisms (GMO) detection in food

EQUIPMENT AND REAGENTS:

Equipment and reagents - not supplied with the kit	
Equipment	Description
Thermal cycler	
Water bath adjustable to 35°C	
Centrifuge for LCD-Array	Cat. No HS-500-01,Chripron GmbH
Micro pipettes	Range from 2 µl to 1000 µl
Optional equipment	
Equipment	Description
Slide Scanner	cat. no HS-300-01, Chipron GmbH
SlideReader Analysis Software	cat. no HS-200-01, Chipron GmbH
Reagent and Materials	
Reagents for DNA extraction	
PCR chemicals	
Taq Polymerase, Buffer, dNTPs	
PCR grade water	
Deionised water	
Disposable gloves	
Sterile filter tips	
PCR reaction vessels	
3 wash containers	
1l- bottles	

METHOD DESCRIPTION:

The test consists of two main procedure steps:

1. Isolation of DNA from food sample (of plant origin, containing soy or maize)
2. PCR amplification of DNA usually used in vectors for plant transformation (i.e. Cauliflower mosaic virus 35S promoter (35S CaMV) and nopaline synthase terminator from Agrobacterium tumefaciens (Tnos).

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DNA isolation from food sample

A special kit designed for rapid purification of DNA from raw or processed food of plant, animal or mixed origin will be used for the isolation of DNA from Genetically Modified Organisms (GMO) in the food. Purified DNA, isolated via this method, is free of contaminants e.g. proteins, lipids, dyes, detergents, organic inhibitors of enzymatic reactions, buffers, salts, divalent cations, among others. Thus the DNA is ready for PCR screening of DNA from Genetically Modified Organisms.

PCR

Taq PCR Master Mix will be applied for the identification of DNA derived from GMO in food samples. PCR kit is a ready-to-use solution containing Taq DNA Polymerase, optimized reaction buffer, MgCl2 , dNTPs and two gel tracking dyes. Use of Color Taq PCR Master Mix (2x) allows to save time and reduce contamination risk due to fewer pipetting steps during PCR set-up. Taq DNA Polymerase is a thermostable enzyme from Thermus aquaticus. The enzyme replicates DNA at 74°C and exhibits a half-life of 40 min at 95°C. Catalyzes the polymerization of nucleotides into duplex DNA in the 5'→3' direction in the presence of magnesium ions. Maintains the 5'→3' exonuclease activity and lacks the 3'→5' exonuclease activity. Adds extra A at the 3' ends. Color Taq PCR Master Mix (2x) is recommended for use in PCR and primer extension reactions at elevated temperatures to obtain a wide range of DNA products up to 10 kb.

The products of PCR will be electrophoretically separated in 0.8% agarose in the presence of ethidium bromide. The presence of the genes derived from GMO will be investigated by detection of fragments of 35S CaMV promoter and Nos Terminator.

ARRAY DESCRIPTION:

Each LCD-Chip contains eight identical arrays in rectangular reaction chambers which can be addressed individually. Functional controls to monitor hybridisation, secondary labeling and staining are located in three corners. The arrays of the MEAT 5.0 kit consist of an 8 x 8 pattern with average spot diameters of 350 µm. The species-specific capture probes are positioned as vertical duplicates.

INTERPRETATION OF THE RESULTS:

1. **General remarks**

GMOs will be detected in tested food products (such as flour, cereal, snack, sausages etc.) Food samples will be used as material for DNA extraction and PCR amplification. The presence of Cauliflower mosaic virus 35S promoter (P35S) or nopaline synthase

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terminator from Agrobacterium tumefaciens (Tnos) indicates the food contains transgenic components.

2. **Methods of analysis**

The formation of dark visible precipitates at positions (spots) where DNA/DNA hybridisation between the PCR amplicons and the immobilized capture probes took place, combined with the crystal clear polymer support (LCD chip), offers the opportunity to use two different analysis methods. Simple optical examination (naked eye, with or without magnifying lenses) or transmission light scanning followed by software-assisted image analysis (details are given below). The experiments of all validations and performance tests of the product have been analysed with both methods in parallel with identical results. Regardless of the method chosen for analysis, negative controls should always be included into the experimental scheme to ensure that no artificial background 'signals' are detected due to cross contamination or 'over staining'.

LITERATURE:

Pacheco Coello, R., Pestana Justo, J., Factos Mendoza, A. et al. 2017. Comparison of three DNA extraction methods for the detection and quantification of GMO in Ecuadorian manufactured food. BMC Res Notes 10, 758. https://doi.org/10.1186/s13104-017-3083-x

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43. Identification of polyphenolic compounds in plant raw materials by the UHPLC-MS method

EQUIPMENT AND REAGENTS:

Equipment and reagents - not supplied with the kit	
Equipment	Description
ACQUITY Ultra Performance LC system	photodiode array detector with a binary solvent manager (Waters Corporation, Milford, MA, USA) with a mass detector G2 Q-Tof micro mass spectrometer (Waters, Manchester, UK) equipped with an electrospray ionization (ESI) source operating in negative and positivemode.
Sonic bath	300 W, 40 kHz; Sonic 6D, Polsonic, Warsaw, Poland
Centrifuge	19,000g for 10 min
Pipettes	Range from 2 µl to 1000 µl
Optional equipment	
Equipment	Description
Freeze-dryer	24 h; Christ Alpha 1–4 LSC; Martin Christ GmbH, Osterode am Harz, Germany
laboratory mill	IKA 11A; Staufen, Germany
hydrophilic PTFE 0.20 mm membrane	Millex Samplicity Filter, Merck
Reagent and Materials	
methanol	
ascorbic acid	
acetic acid	
formic acid	
acetonitrile	
reference compounds of polyphenols	

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METHOD DESCRIPTION:

The method consists of two main procedure steps.

- Extraction of phenolic compounds from raw materials
- Separation of polyphenols on a chromatographic column and detection in a DAD detector

Extraction

The lyophilized and powdered samples of plant raw material are extracted with 5 mL of 30% methanol acidified with 1% acetic acid and containing 1% ascorbic acid as antioxidant. The extraction is performed by incubation for 20 min under sonication and with occasional shaking. Next, the slurry is centrifuged at 19,000 × *g* for 10 min, and the supernatant is filtered through a hydrophilic PTFE 0.20 µm membrane (Millex Simplicity Filter, Merck) and used for analysis.

Separation / Detection

Identification of polyphenols is carried out using an ACQUITY Ultra Performance LC system equipped with a photodiode array detector with a binary solvent manager (Waters Corporation, Milford, MA, USA) with a mass detector G2 Q-Tof micro mass spectrometer (Waters, Manchester, UK) equipped with an electrospray ionization (ESI) source operating in negative mode. Separations of individual polyphenols is carried out using a UPLC BEH C18 column (1.7 µm, 2.1 × 100 mm, Waters) at 30 °C. The samples (10 µL) are injected, and the elution is completed in 15 min with a sequence of linear gradients and constant flow rates of 0.42 mL min^{-1}. The mobile phase consists of solvent A (0.1% formic acid, v/v) and solvent B (100% acetonitrile). The linear gradient is as follows: 0.0-1.0 min, 99% A, 0.42 mL/min (isocratic), 1.0-12.0 min, 65.0% A, 0.42 mL/min (linear), 12.0-12.5 min, 99% A, 0.42 mL/min (linear), 12.5-13.5 min, 99% A, 0.42 mL/min (isocratic). The analysis is carried out using full-scan, data-dependent MS scanning from m/z 100 to 1500. Leucine encephalin is used as the reference compound at a concentration of 500 pg/µL, and the $[M-H]^-$ ion at 554.2615 Da and $[M+H]^+$ion at 556.2771 are detected. The $[M-H]^-/[M+H]^+$ ions are detected during a 15 min analysis performed within ESI-MS accurate mass experiments, which are permanently introduced via the Lock Spray channel using a Hamilton pump. The lock mass correction is ±1.000 for the mass window. The mass spectrometer is operated in negative-ion and positive-ion modes, set to the base peak intensity (BPI) chromatograms, and scaled to 12,400 counts per second (cps) (100%). The optimized MS conditions are as follows: capillary voltage of 2500 V, cone voltage of 30 V, source temperature of 100 °C, desolvation temperature of 300 °C, and desolvation gas (nitrogen) flow rate of 300 L/h. Collision-induced fragmentation experiments are performed using argon as the collision gas, with voltage ramping cycles from 0.3 to 2 V.

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INTERPRETATION OF THE RESULTS:

Characterization of the single components is carried out via the retention time and the accurate molecular masses. Each compound is optimized to its estimated molecular mass in the negative/positive mode, before and after fragmentation. The data obtained from UPLC-MS are subsequently entered into the MassLynx 4.0 ChromaLynx Application Manager software (Waters). Based on these data, the software is able to scan different samples for the characterized substances. The runs are monitored at the following wavelengths: ellagitannins and ellagic acid derivatives at 254 nm, flavan-3-ols and hydroquinones at 280 nm, phenolic acids at 320 nm, flavones at 340 nm, flavonol glycosides at 360 nm and anthocyanins at 520 nm. The PDA spectra are measured over the wavelength range of 200−600 nm in steps of 2 nm. The retention times and spectra are compared to those of the authentic standards.
The quantification of phenolic compounds is performed by external calibration curves (R^2 = 0,999), using reference compounds selected based on the principle of structure-related target analytic/standard (chemical structure or functional group).

DEVICE PERFORMANCE

The method is validated in accordance with the requirements for new methods for linearity, limit of detection (LOD), limit of quantification (LOQ), precision (interday and intraday precision), repeatability and stability.

1. Linearity

Standard calibration curves were prepared using the following standards: apigenin 7-O-glucoside, arbutin, caffeic acid, 3-caffeoylquinic, 4-caffeoylquinic and 5-caffeoylquinic acid, (+)-catechin, p-coumaric, ferulic, sinapic and gallic acid, isorhamnetin 3-O-rutinoside and 3-O-glucoside, kaempferol 3-O-glucoside, procyanidin A2 and B2, quercetin 3-O-galactoside, 3-O-glucoside and 3-O-rutinoside, cyanidin 3-Ogalactoside, cyanidin 3-O-arabinoside, cyanidin 3-O-rutinoside, and ellagic acid. Standard stock solutions were diluted to appropriate concentrations (five calibration points were used in each case) for the plotting of calibration curves. The linearity was obtained by plotting the peak areas versus the corresponding concentrations (ppm) of each analyte.

2. LODs and LOQs

The LOD and LOQ of standard stock solutions were determined by preparing dilute solutions of standards (five dilution points were used in each case) and injecting these solutions into the liquid chromatograph and recording the signal-to-noise (S/N) ratio for peaks at each concentration. LODs and LOQs were determined at an S/N ratio of about 3 and 10, respectively.

3. Precision, repeatability, and stability

For the intraday precision test, the standard solution containing the standard compounds were analyzed three times within 1 day (n=3), while for the interday precision test, the standard solution was examined each day for 3 consecutive days (n=9). Intraday and interday precision of the current method was evaluated by calculating the relative standard deviation

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(RSD, %) of the peak areas. For repeatability, three different sample solutions (n=3) prepared from the same sample were analyzed, and variations were expressed by RSD (%).

LITERATURE:

1. Kolniak-Ostek, J. 2015. Identification and quantification of polyphenolic compounds in ten pear cultivars by UPLC-PDA-Q/TOF-MS. Journal of Food Composition and Analysis, 49, 65–77

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44. Inorganic arsenic speciation

EQUIPMENT AND REAGENTS:

Equipment and reagents - not supplied with the kit	
Equipment	Description
Water bath	Adjustable to 100 °C
Mortar grinder	Retsch model RM100 (Haan, Germany)
Micro pipettes	Range from 1 to 5 mL
Hot-air oven	Adjustable to 70 °C
HPLC	high-performance liquid chromatography (HPLC) system consisting of a Varian 9012 ternary pump (Varian, San Fernando, USA), a Rheodyne 7125 injector and a 50 µL loop for sample introduction
Column	Hamilton PRP-X100 anion-exchange column (10 µm, 250 mm × 4.1 mm; Hamilton, Reno, USA).
Pre-column	Pre-column packed with the same material (12–20 µm; 25 mm × 2.3 mm; Hamilton, Reno, USA).
Peristaltic pump	Gilson Miniplus 3 peristaltic pump
Lamp	Boosted-discharge hollow cathode lamp (Photron Pty. Ltd, Victoria, Australia
Atomic fluorescence spectrophotometer	PSA 10.044 Excalibur, PS Analytical, Kent, UK
Reagent and Materials	
Ultrapure deionized water, 18 M	Milli-QR system
Disposable gloves	
Trifluoroacetic acid (TFA)	Sigma-Aldrich, St. Louis, MO, U.S.A
$NaAsO_2$ and $Na_2HAsO_4{\cdot}7H_2O$	Panreac and Chem Service
$NaBH_4$	Panreac
HCl	Panreac

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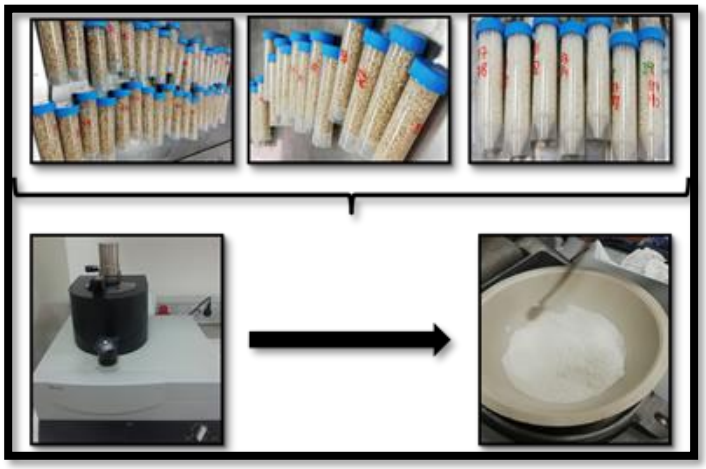
METHOD DESCRIPTION:

Sample preparation

All samples were milled with a mortar grinder with this procedure the final particle size of the samples was below 10 µm. Later, samples were dried at 70 °C until constant weight in a hot-air oven.



Extraction of arsenic species

Approximately 0.5–2.0 g of milled and dried samples was treated with 3 mL of 2 M TFA (trifluoroacetic acid). The mixture was allowed to stand for 6 h at 100 °C in a 50 mL capped HDPE (High-Density PolyEthylene) centrifuge tube. The mixture was centrifuged, and the supernatant collected and diluted to proper volume with deionized water (Milli-Q® system) and, then, filtered through a 0.45 µm nylon syringe filter.

External calibration

External calibration was accomplished using standards of 10, 20, 30, 40, and 50 µg L^{-1} of each of the two As species studied (arsenite: $NaAsO_2$ and arsenate: $Na_2HAsO_4{\cdot}7H_20$). Analysis was run in triplicate.

High-performance liquid chromatography (HPLC) conditions

A high-performance liquid chromatography (HPLC) system consisting of a Varian 9012 ternary pump (Varian, San Fernando, USA), a Rheodyne 7125 injector and a 50 µL loop for sample introduction, was used. Separations of As species [arsenite and arsenate] were performed on a Hamilton PRP-X100 anion-exchange column (10 µm, 250 mm × 4.1 mm i.d.; Hamilton, Reno, USA). A guard column packed with the same material (12–20 µm; 25 mm × 2.3 mm i.d.)

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preceded the analytical column. It was carried out in 15 min in the anion-exchange column using phosphate buffer pH 5.95 as the mobile phase at a 0.800 mL min^{-1} flow rate. The elution order was arsenite (As III) and arsenate (As V).

Hydride generation of volatile arsines (HG) & atomic fluorescence spectrometer (AFS) conditions

Hydride generation of volatile arsines prior to the detection was performed adding on-line solutions of HCl and $NaBH_4$ by means of a Gilson Miniplus 3 peristaltic pump. The quantification of As species was performed on a hydride generation system (PSA 10.044, PS Analytical, Kent, UK) using an atomic fluorescence spectrometer (AFS) system (PSA 10.044 Excalibur, PS Analytical, Kent, UK) equipped with a boosted-discharge hollow cathode lamp (Photron Pty. Ltd, Victoria, Australia). The analogical signal output was connected to a computer equipped with chromatographic software (PS Analytical, Kent, UK). The limit of quantification was 0.004 mg/kg^{-1}.

Reference material

As to quality assurance step, the CRM ERMR-BC211 (CYMIT Química S.L., Barcelona, Spain), "rice flour," was directly provided by the Joint Research Centre of the European Commission and analyzed for As speciation. The experimental results obtained for this CRM should be compared to certified values.

INTERPRETATION OF THE RESULTS:

1. **General remarks**

Next Figure shows a chromatogram in which there are 4 peaks, the first of them (retention time of 4.24 min) is As III while the last peak (11.73) is As V. The rest of the peaks are organic arsenic species. For the calculation, it is necessary to know the areas of each of the inorganic spices and the sum of them will be the inorganic arsenic (As-i).

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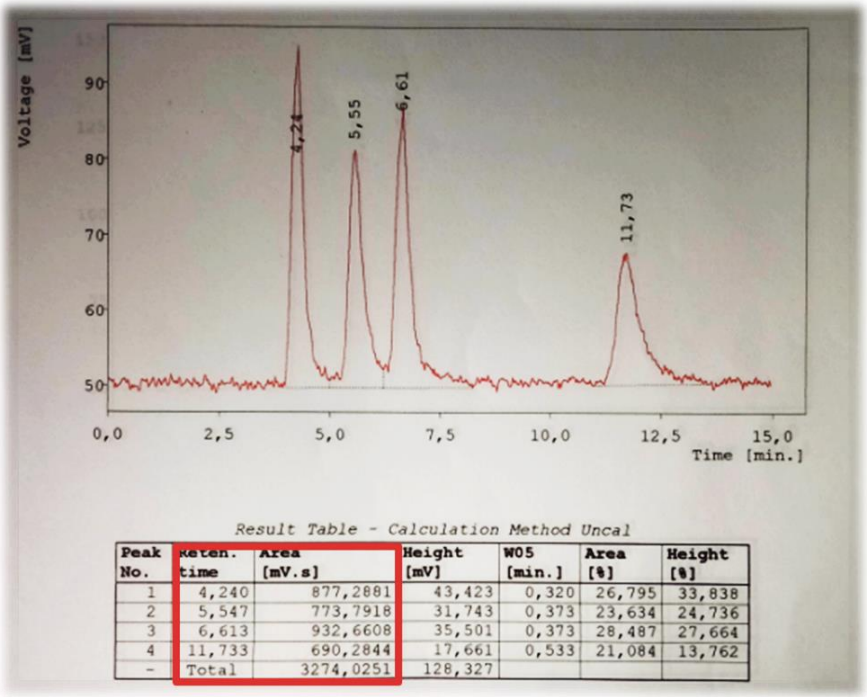
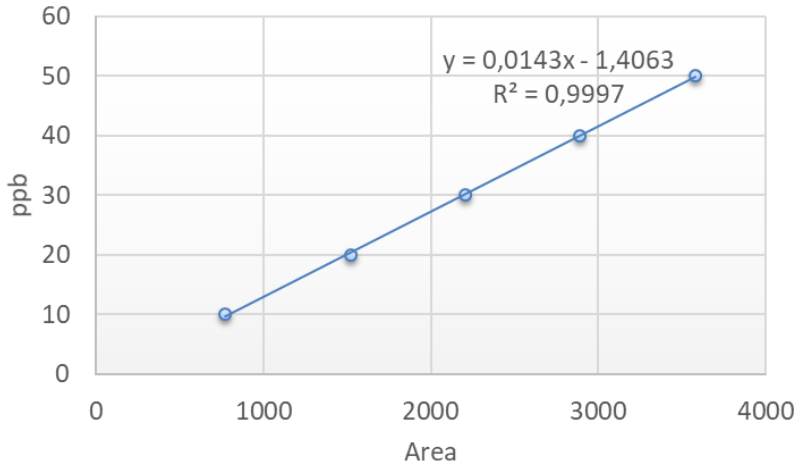


Result Table - Calculation Method Uncal

Peak No.	Reten. time	Area [mV.s]	Height [mV]	W05 [min.]	Area [%]	Height [%]
1	4,240	877,2881	43,423	0,320	26,795	33,838
2	5,547	773,7918	31,743	0,373	23,634	24,736
3	6,613	932,6608	35,501	0,373	28,487	27,664
4	11,733	690,2844	17,661	0,533	21,084	13,762
-	Total	3274,0251	128,327			

The following graph shows the standard line of one of the inorganic species of interest. It has been represented from the areas obtained from the chromatograms and the known concentration. From this equation, the area obtained from the test sample will be included. Taking the weight and the volume into account, we will obtain the value of the concentration.



RECOMMENDATIONS

In general, for the analysis of metallic traces, some recommendations should be considered:

- extreme precautions regarding the cleaning of the material (all glassware was treated with 10% HNO3 for 24 h and then cleaned 3 times with deionized water before use);
- reagents must be extremely pure, metal-free;
- reduce number of steps required for determination; and,
- perform at least three repetitions contrasting with other laboratories.

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LITERATURE:

1. Gavilanes-Terán I, Cano-Lamadrid M, Idrovo-Novillo J, García-García E, Veloz-Mayorga N, Erazo-Arrieta R, Burló F, Cruz-Paca F, Carbonell-Barrachina ÁA. 2019. Inorganic arsenic content in Ecuadorian rice-based products. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. June; 36(6) pp.922-928. doi: 10.1080/19440049.2019.1595744. Epub 2019 Apr 22. PMID: 31009318.
2. Cano-Lamadrid M, Munera-Picazo S, Burgos-Hernández A, Burló F, Carbonell-Barrachina ÁA. 2016. Inorganic and Total Arsenic Contents in Rice and Rice-Based Foods Consumed by a Potential Risk Subpopulation: Sportspeople. J Food Sci. Apr;81(4):T1031-7. doi: 10.1111/1750-3841.13269. Epub 2016 Mar 14. PMID: 26990091.
3. Cano-Lamadrid M, Munera-Picazo S, Burló F, Hojjati M, Carbonell-Barrachina ÁA. 2015. Total and inorganic arsenic in Iranian rice. J Food Sci. May;80(5):T1129-35. doi: 10.1111/1750-3841.12849. Epub 2015 Mar 28. PMID: 25817563.
4. Cano-Lamadrid M, Girona D, García-García E, Dominguis-Rovira V, Domingo C, Sendra E, López-Lluch D, Carbonell-Barrachina Á.A. 2020. Distribution of essential and non-essential elements in rice located in a Protected Natural Reserve “Marjal de Pego-Oliva”, Journal of Food Composition and Analysis, Volume 94, 103654. doi: 10.1016/j.jfca.2020.103654

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Universidad Miguel Hernández de Elche, Spain
Prof. Francisco Burló Carbonell
Marina Cano Lamadrid. Junior researcher

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45. Meat 5.0 LCD array kit, DNA - based identification of animal species

EQUIPMENT AND REAGENTS:

Equipment and reagents - not supplied with the kit	
Equipment	Description
Thermal cycler	
Water bath	Adjustable to 35 °C
Centrifuge for LCD-Array	Cat. No HS-500-01,Chripron GmbH
Micro pipettes	Range from 2 µl to 1000 µl
Optional equipment	
Equipment	Description
Slide Scanner	cat. no HS-300-01, Chipron GmbH
SlideReader Analysis Software	cat. no HS-200-01, Chipron GmbH
Reagent and Materials	
Reagents for DNA extraction	
PCR chemicals	Taq Polymerase, Buffer, dNTPs
PCR grade water	
Deionised water	
Disposable gloves	
Sterile filter tips	
PCR reaction vessels	
3 wash containers	
1l- bottles	

METHOD DESCRIPTION:

The test consists of two main procedure steps.

- PCR amplification of DNA fragments with biotin incorporation
- Hybridisation of PCR fragments to LCD-Arrays and detection

PCR

One primer mix is provided with the kit. The multiple primer pairs in this mix target a portion of the 16S RNA gene of a broad range of Vertebrate species. When using the primer mix 'MEAT' distinct amplification products of 115 – 125 bp can be expected.

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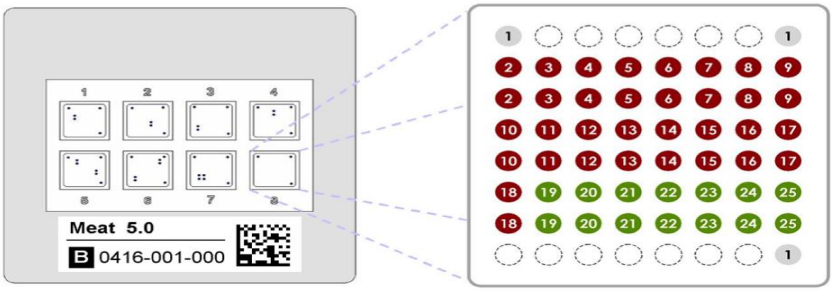
Hybridisation / Detection

The labelled PCR fragments are combined with the hybridisation buffer (provided) and hybridised to the individual array fields of one chip. During hybridisation the labelled PCR fragments will bind to the specific capture probes immobilized as spots on the bottom of each field. Following a short washing procedure, each field is incubated with a secondary label solution (enzyme-conjugate). After a second washing step, the positions (spots) where PCR fragments and secondary label are bound can be visualized as blue precipitate formed by the enzyme substrate provided as "STAIN". The data read-out can either be done by simple 'naked-eyed' examination, using the 'Analysis Matrix' provided with the kit or, alternatively, with the scanner and software from the "Analysis-Package" which can be obtained from Chipron GmbH.

For experienced users the whole procedure will take 3-4 hrs (depending on the duration of the PCR amplification).

ARRAY DESCRIPTION:

Each LCD-Chip contains eight identical arrays in rectangular reaction chambers which can be addressed individually. Functional controls to monitor hybridisation, secondary labeling and staining are located in three corners. The arrays of the MEAT 5.0 kit consist of an 8 x 8 pattern with average spot diameters of 350 µm. The species-specific capture probes are positioned as vertical duplicates.



No.	Name	*Species*	No.	Name	*Species*
01	Hyb-Ctrl	Hybridization Control			
02	Cattle	*Bos taurus, Bos bison*	14	Red Deer *	*Cervus elaphus*
03	Sheep	*Ovis aries*	15	Fallow Deer	*Dama dama*
04	Equine	*Equus caballus, E. asinus*	16	Springbok	*Antidorcas marsupialis*
05	Goat	*Capra hircus*	17	Canine / Dog	*Canis sp.*
06	Camels	*Camelus sp.*	18	Cat	*Felis silvestris*
07	Buffalo	*Bubalus bubalis*	19	Chicken	*Gallus gallus*
08	Pork	*Sus scrofa*	20	Turkey	*Meleagris gallopavo*
09	Kangaroo	*Macropus rufus / giganteus*	21	Goose	*Anser sp.*
10	Hare	*Lepus europaeus*	22	Ostrich	*Struthio camelus*
11	Rabbit	*Oryctolagus cuniculus*	23	Mallard Duck	*Anas platyrhyncos*
12	Reindeer *	*Rangifer tarandus*	24	Muscovy Duck	*Cairina moschata*
13	Roe Deer	*Capreolus capreolus*	25	Pheasant	*Phasianus sp.*

* Weak cross reactivity of the capture probe for Reindeer with pure Red Deer and vice versa can occur at high target concentrations.

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INTERPRETATION OF THE RESULTS:

2. **General remarks**

LCD-Arrays generate qualitative results indicating the 'presence' or 'absence' of the respective parameter (species) within the sample material used for DNA extraction and PCR amplification. Since the assay principle is based on DNA detection, vital and dead organisms will be identified likewise. 'Presence' and 'absence' in this respect is defined by the assay specific detection range and limits for eachparameter. Although different signal intensities can be observed during the analysis of LCD-Arrays and these intensities are generally correlated with the amount of target copies in the starting material, it should be noted that LCD-Arrays have not been validated as tools for absolute quantification.

3. **Methods of analysis**

The formation of dark visible precipitates at positions (spots) where DNA/DNA hybridisation between the PCR amplicons and the immobilized capture probes took place, combined with the crystal clear polymer support (LCD chip), offers the opportunity to use two different analysis methods. Simple optical examination (naked eye, with or without magnifying lenses) or transmission light scanning followed by software-assisted image analysis (details are given below). The experiments of all validations and performance tests of the product have been analysed with both methods in parallel with identical results. Regardless of the method chosen for analysis, negative controls should always be included into the experimental scheme to ensure that no artificial background 'signals' are detected due to cross contamination or 'over staining'.

DEVICE PERFORMANCE

All evaluation experiments with respect to the assay specificity and sensitivity have been performed with 'Animal Control DNA' extracted from skeletal muscle tissues of the respective species at Chipron GmbH. DNAs have been object to Sanger Sequencing of fragments of the mitochondrial cytochrome b (cyt b) or cytochrome c oxidase subunit I (cox I) genes for species confirmation and have been treated with RNase A prior to quantification and standardization to stock concentrations of 100 ng/µl.

LITERATURE:

1. Iwobi A., Huber I., Hauner G., Miller A. and Busch U. 2011. Biochip Technology for the Detection of Animal Species in Meat Products; Food Analytical Methods, Vol. 4, No. 3, pp. 389-398.
2. Cawthorn D.M., Steinman H.A., Hoffman L.C. 2013. A high incidence of species substitution and mislabelling detected in meat products sold in South Africa; Food Control 32, pp. 440 – 449.

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46. Method for simultaneous determination of PAHs, PCB and Dioxin using Solid Phase Extraction -SPE- followed by GC-ECD and GC-IT/MS

EQUIPMENT AND REAGENTS:

Equipment	
Equipment	Description
Gas chromatograph (GC) TraceGC (ThermoFischer, Milan, Italy)	Separation of analytes based on chemical and physical characteristics.
Ion Trap Mass Spectrometry (IT/MS) PolarisQ (ThermoFischer, Milan, Italy)	Qualitative-quantitative analysis.
Gas chromatograph (GC) Dani 8610	Separation of analytes based on chemical and physical characteristics.
ECD with radioactive sorgent ^{63}Ni	Qualitative-quantitative analysis.
Reagent and Materials	
Chemical standard: Naphthalene (Na); Acenaphthylene (Acy); Acenaphthene (Ace); Fluorene (Fl); Phenanthrene (Phe); Anthracene (Ant); Fluoranthene (Fu); Pyrene (Py); Benzo[a]anthracene (BaA); Chrysene (Chr); Benzo[b+j]fluoranthene (Bb+jF); Benzo[k]fluoranthene (BkF); Benzo[e]pyrene (BeP); Benzo[a]pyrene (BaP); Perylene (Per); Dibenz[a,h]anthracene (DahA); Indeno[1,2,3-cd]pyrene (Ipy); Benzo[ghi]perylene (BghiP); Dibenzo[a,l]pyrene(DalP); Dibenzo[a,e]pyrene(DaeP); Dibenzo[a,i]pyrene (DaiP); Dibenzo(a,h)pyrene (DahP). Internal standard: deutered standard Dilution solvent: Acetone. Conditioning solvent: UP water, acetone, Ethanol and Hexane. Extraction solvent: Hexane, Ethyl acetate, Acetone, Acetonitrile, Benzene, Chloroform, Dichloromethane, Ethanol, Isoctaneand Toluene.	2-chlorobiphenyl (BZ # 1) 2,3-dichlorobiphenyl (BZ # 5) 2,4,5-trichlorobiphenyl (BZ # 29) 2,2,4,6-tetrachlorobiphenyl (BZ # 50) 2,2,3,4,5-pentachlorobiphenyl (BZ # 87) 2,2,4,4,5,6-hexachlorobiphenyl (BZ # 154) 2,2,3,4,5,6,6-heptachlorobiphenyl (BZ # 188) 2,2,3,3,4,5,6,6-octachlorobiphenyl (BZ # 200) decachlorobiphenyl (BZ # 209) 1,2,3,4-tetrachlorodibenzo-p-dioxin 1,2,3,4,7-pentachlorodibenzo-p-dioxin 1,2,3,4,7,8-hexachlorodibenzo-p-dioxin 1,2,3,4,6,7,8-heptachlorodibenzo-p-dioxin SPE cartridge with Carbograph, C18, C8, XAD2, XAD7, -NH_2, -OH solid phase. Other chemical: Sodium chloride, N_2.

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METHOD DESCRIPTION:

Preparation of standard solutions

For the development of the method and the study of the method parameters, solutions of known concentrations of the standards in question were made. Specifically, 10 mg of each PAHs were weighed by analytical balance and solubilized in 10mL of acetone. The preparation of the internal standard (I.S.) solution was done by weighing 1 mg of Phenanthrene D10solubilized in 10 mL of acetone (100 µg g^{-1}).

Preparation of calibration curves

The mother solutions have been diluted with acetone to set up a PAHs/PCBs/Dioxins mix solution. Appropriate dilution of the PAHs/PCBs/Dioxins mix solution, 10 µg g^{-1}, with acetone to obtain seven solutions of known concentrations (0.05, 0.5, 1.0, 2.5, and 5.0 µg g^{-1}).

Study of adsorption isotherms curves

For the evaluation of the adsorbent phase-analyte interaction under static conditions, the adsorption isotherms are performed. For their determination, five solutions of 100 mL at known concentration were prepared. The solutions of PAHs/PCBs/Dioxins are made with the addition of 25 gL^{-1} of NaCl to control the salting out effect.

50 mL of each solution is used as the reference solution (head solution) and 50 mL as the test solution (tail solution). The latter is put in contact with 50 mg of suitable specific phase in a flask for about 24 hours, at a constant temperature, in the dark and under slight stirring. Subsequently, the solid phase is separated from the liquid phase by filtration. The obtained liquid phase and the reference heads are extracted with 1mL hexane under magnetic stirring for 5 minutes. After stirring, the hexane is separated from the aqueous phase and brought up to about 200 µL under a gentle flow of nitrogen. Finally, IS is added and 1 µL of this solution was injected into the GC-IT/MS instrument.

Cartridge packing

SPE cartridges are prepared by the suspension packing method using100 mg of suitable specific phase and three solvents of different polarity. In order, 5 mL of hexane, 5 mL of acetone, and 5 mL of ethanol are used. Finally, once the SPE cartridge has been packed, 5 mL of ultrapure H_2O is passed through it to prepare the SPE for the passage of the aqueous solution containing the PAHs/PCBs/Dioxins.

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Study of breakthrough curves

Breakthrough curves are constructed to evaluate the adsorbent phase-analyte interaction under dynamic conditions. The solutions are prepared by controlling pH, ionic strength, and temperature. We proceed with the elution of fractions of 100 mL each having the characteristics mentioned above with an average elution flow between 4-8 $mL.min^{-1}$. Each fraction, including the reference one, is extracted with 1 mL of hexane with a magnetic stirrer and a magnetic stir bar for 5 min. The hexane is concentrated to about 200 µL under the same conditions as the isotherms and the internal standard is added. Finally, 1 µL of this solution was injected into the GC-IT/MS instrument.

Study of recovery solvent

The enrichment step was carried out through the elution for each SPE cartridge, containing 100 mg of suitable specific phase of an aqueous solution of 100 mL of PAHs/PCBs/Dioxins mix having a concentration of 10 ppb with 25 $g.L^{-1}$ of NaCl according to a flow rate between 4-8 $mL.min^{-1}$. The recovery test was carried out by making a different solvent flow into the cartridge for each test. Recovery was performed with an initial 4 mL fraction and two subsequent 2 mL fractions for a total of 8 mL for each solvent. Each recovery, collected in a vial, was brought under a light flow of nitrogen to about 200 µL and the internal standard was added. Finally, 1 µL of this solution was injected into the GC-IT/MS instrument.

Example of analysis procedure on a sample of honey

For each real sample, 1 g of honey was dissolved in 100 mL of aqueous solution containing 25 $g\ L^{-1}$ of NaCl. This solution was passed into a SPE cartridge containing 100 mg of Carbograph 1 with an average flow rate of 4-8 $mL.min^{-1}$. The analytes were subsequently extracted with 5 mL of toluene which was brought up to 200 µL and the internal standard was added. Finally, 1 µL of this solution was injected into the GC-IT/MS instrument.

Study of method parameters

The analytical protocol was validated in terms of linearity range, correlation coefficients, reproducibility, intra- and inter-day errors and recoveries, and by performing the entire procedure on honey samples.

INTERPRETATION OF THE RESULTS:

The search of PAHs/PCBs/Dioxins was performed using gas chromatography coupled to ion trap mass spectrometry. The latter allowed for a better cleaning of the chromatogram through the acquisition of data in SIM and the confirmation of the analyte through the database. Quantification was performed via calibration curves using the internal standard method to eliminate injection volume error. The results were expressed as $\mu g\ g^{-1}$.

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DEVICE PERFORMANCE

The analytical instrumentation supplied in our laboratory is constantly updated. All the tools are automated and require the use of specific software and qualified personnel.

LITERATURE:

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47. Method for different classes of phospholipids analysis using liquid chromatography with charge aerosol detector (CAD)

EQUIPMENT AND REAGENTS:

Equipment	Description
Water bath	Adjustable to 100 °C
Rotary shaker	
Centrifuge	
Rotary vacuum evaporator	
SPE system	CHROMABOND SPE vacuum manifold for 12 positions, silica gel columns (Discovery DSC-Si SPE 500 mg)
HPLC	Ultimate 3000 from DIONEX chromatograph equipped with DGP-3600A dual-pump fluid control module, a TCC-3200 thermostated column compartment, an WPS-3000 autosampler and the Corona Charged Aerosol Detector (CAD).
Columns for HPLC	Thermo Betasil DIOL (5 µm, 150 x 4.6 mm) equipped with a precolumn containing the same sorbents
Reagent and Materials	
chloroform, methanol	analytical grade from POCh (Poland, Gliwice)
saline solution (0.05M of NaCl)	NaCl pure p. a. from POCh (Poland, Gliwice)
Solvents for HPLC	Hexane, 2-propanol, formic acid, all of chromatographic grade (Merck)
Standards for HPLC	L-α-phosphatidylcholine (PC) (purity ≥ 99%), L-α-lysophosphatidylcholine (LPC) (purity ≥ 99%), L-α-phosphatidylethanolamine (PE) (purity ≥ 97%) and L-α-phosphatidyl-L-serine (PS) (purity ≥ 97%) were purchased from Sigma-Aldrich Chemical Co. L-α-phosphatidylinositol (PI) (purity ≥ 97%) was purchased from Larodan.

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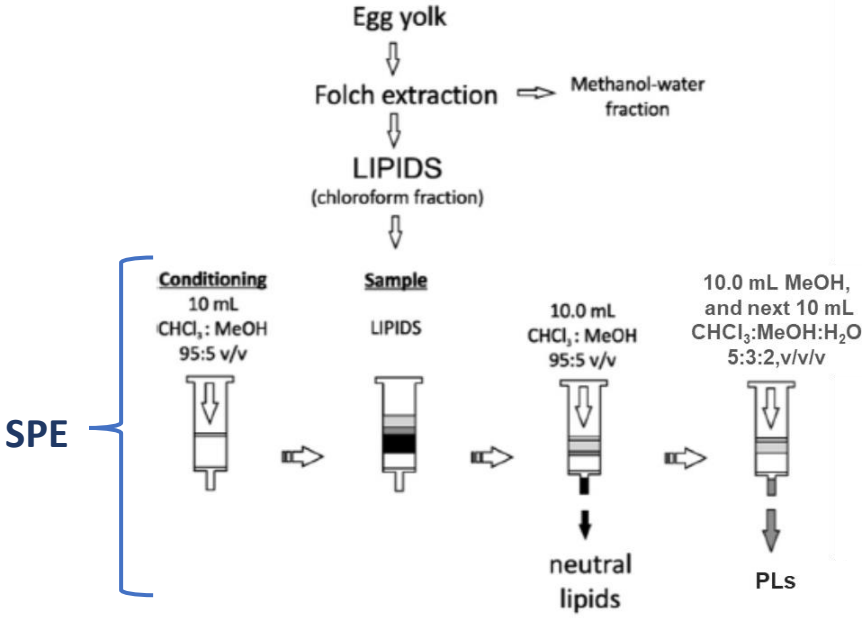
METHOD DESCRIPTION:



Sample preparation and extraction of lipid fraction

Egg yolk lipids or milk lipids were isolated as a crude lipid fraction by extraction according to Folch et al. Prior to extractions, milk samples were warmed to 35 °C. Samples of egg yolk or milk (10 g or 10 mL, respectively) was dissolved in 200 mL chloroform:methanol (2:1, v/v) and 20 mL of saline solution (0.05M of NaCl), shaken vigorously for 15 min on a rotary shaker (200 rpm), and centrifuged at 5000 rpm for 5 min. After centrifugation the lower chloroform layer was released and the process was repeated adding after 140 mL of chloroform to the upper (methanol-water) phase. The two chloroform phases were pooled and evaporated in vacuo. Separated lipids were exposed to a stream of N_2 and frozen at -20°C until further analysis.

Solid-phase extraction (SPE)

SPE column was conditioned with 10 mL of chloroform: methanol (95:5, v/v), and then the crude lipid extract (100 mg) dissolved in 1.0 mL of chloroform:methanol (95:5, v/v) was applied to the SPE cartridge. The neutral lipids were eluted with 20 mL of chloroform: methanol (95:5, v/v). The recovery of PLs was performed by using of 10 mL of methanol and then 10mL of chloroform: methanol:water (5:3:2,v/v/v). The phospholipid fractions were evaporated using a rotary vacuum evaporator at 45°C and redissolved in 1 mL of chloroform: methanol (2:1, v/v) for HPLC analysis.

High-performance liquid chromatography (HPLC) conditions

HPLC analysis was carried out using a Betasil DIOL 5 µm (150 × 4.6mm) column equipped with a precolumn containing the same sorbents. The injection volume was 10 µL in all experiments and the cooling temperature for the samples was 20°C. The gradient elution was used for the separation of phospholipids (Table 1). The gradient had a constant flow 1.5 mL·min^{-1}, with

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solvent A: 13% HCOOH in water; B: hexane; and C: 2-propanol. The column temperature was maintained at 20°C.

Table 1. Solvent gradient elution system required for the elution of phospholipids.

	Percent solvent			Flow (mL/min)
Time (min)	A[a] (%)	B[b] (%)	C[c] (%)	
0.0	3	40	57	1.5
4.0	10	40	50	1.5
9.0	10	40	50	1.5
9.1	3	40	57	1.5
19.0	3	40	57	1.5

[a] **13% Formic acid in water,** [b] **Hexane,** [c] **2-Propanol**

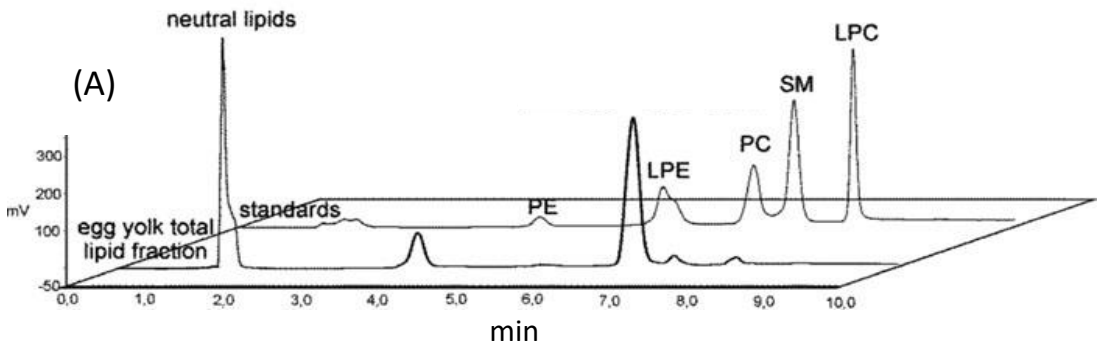
Calibration curves

Calibration curves for each compound were calculated from the area values obtained by injecting 10 µL of chloroform-methanol (2:1, v/v) solutions of PE (0.01–1.67 µg), LPE (0.01–2.17 µg), PC (0.01–2.62 µg), SM (0.01–1.94 µg), LPC (0.01–2.11 µg). At least six standard concentrations of every compound were prepared. Each standard mixture dissolved in chloroform:methanol (2:1, v/v) was injected five times. The resulting peak areas were plotted as a function of the individual phospholipid class amount and fitted using linear model (y=a+bx).

INTERPRETATION OF THE RESULTS:

General remarks

This HPLC-CAD method enables rapid analysis and quantification of six different phospholipid classes (PS, PE, PI, PC, SM and LPC). The following figure 1A shows a chromatogram of a standard mixture and egg yolk total lipid fraction PLs, and figure 1B chromatogram of a standard mixture and milk PLs. The phospholipids components were identified by comparison of their retention times with those of commercial standards.



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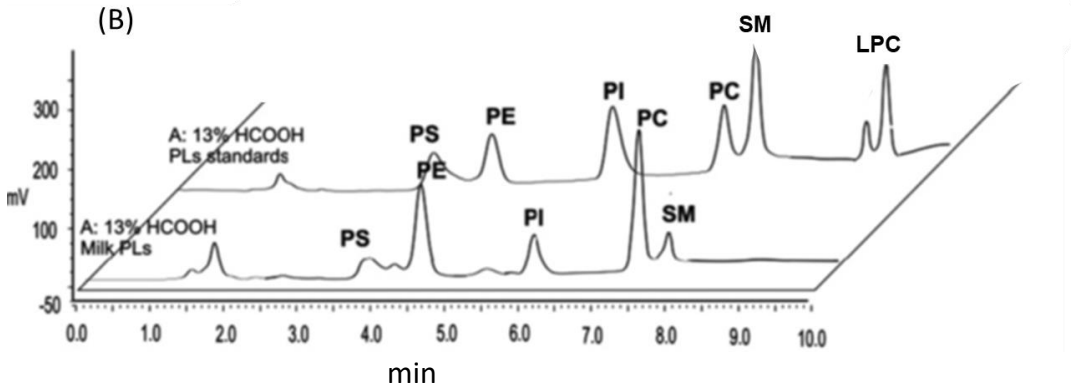

Fig. 1. HPLC-CAD chromatograms of (A) phospholipids standards and egg yolk total lipid fraction and (B) phospholipids standards and milk PLs.

The following table 2 shows the equations of calibration curves in linear model (y=a + bx) for each phospholipid class with the coefficient of determination (R^2). It has been represented from the areas obtained from the chromatograms for standard phospholipids and the known concentration. From this equation, the area obtained from the test sample will be included. Taking the weight and the volume into account, we will obtain the value of the concentration.

Compound	Range (μg)	Linear model ($y=a+bx$)	
		Equation	R^2
PS	0.02–1.66	$40.459(\pm0.27)x-1.133(\pm0.24)$	0.9992
PE	0.02–1.57	$34.476(\pm0.15)x-0.160(\pm0.13)$	0.9996
PI	0.03–2.00	$40.538(\pm0.44)x+1.262(\pm0.47)$	0.9977
PC	0.03–2.16	$25.549(\pm0.18)x+0.422(\pm0.21)$	0.9990
SM	0.03–1.99	$44.567(\pm0.73)x+1.715(\pm0.78)$	0.9948
LPC	0.05–1.44	$27.760(\pm0.23)x+0.388(\pm0.16)$	0.9989

RECOMMENDATIONS

In general, for the quantitative analysis of six classes of phospholipids using HPLC with charge aerosol detector, some recommendations should be considered:

- All the samples should be analyzed consecutively in the same day, for the same analyst to ensure repeatability
- In each case six replicates should be determined.

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LITERATURE:

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48. Methods for determination of hydrosoluble and liposoluble vitamins in vegetables and dairy products

EQUIPMENT AND REAGENTS:

Equipment	
Equipment	Description
HPLC (Dionex, Sunnyvale, CA)	Quaternary pump, injector, DAD spectrophotometer and spectrofluorimeter detector
UV-Spectrophotometer (Perkin Elmer Lambda-25)	Compound and standard quantification
Laboratory miller	Milling/homogenization of samples
Centrifuge	Separation of samples
Ultrasound bath	Degassing of solvents
Freeze-drier (Genesis 25SES freeze dryer, VirTis Co., Gardiner, NY)	To dehydrate/preserve compounds
Reagent and Materials	
Violaxanthin, neoxanthin, anteraxanthin, α-carotene, 9-cis-β-carotene and 13-cis-β-carotene standards (CaroteNature, Lupsingen, Switzerland); lutein, zeaxanthin, and β-cryptoxanthin standards (Extrasynthese, Z.I. Lyon-Nord, Genay, France). All-trans-β-carotene, thiamine and riboflavin standards (Sigma Chemicals, St. Luis, MO, USA). α–, β, γ– and δ–tocopherol standards (Merck, Darmstadt, Germany); α–, β–, γ– and δ-tocotrienol standards purified as reported in Panfili et al. 2003.	

METHOD DESCRIPTION:

Extraction of retinols, carotenoids and tocols

Extraction of retinols, carotenoids and tocols is carried out according to Panfili et al. 1994, 2003, 2004, respectively, with some modifications, depending on analyzed samples. Samples are weighed in a screw-capped tube. Then 5 ml ethanolic pyrogallol (60 g/L), 2/3 mL of absolute ethanol, 1/2 mL of sodium chloride (10 g/L) and 2/3 mL of potassium hydroxide (600 g/L) for alkaline digestion are added. The tubes are flushed with nitrogen, to avoid oxidation. and are put for 45 min in a 70 °C water bath, stirring every 5-10 min. After cooling, 15 mL of sodium chloride (10 g/L) are added. Compounds are extracted with 15 mL of n-hexane/ethyl acetate (9:1, v/v), until the organic layer is colorless. Organic layers are collected and evaporated to dryness. For carotenoid and retinol analysis in normal-phase HPLC (NP), samples are suspended in isopropyl alcohol (10%) in n-hexane. For tocols, samples are dissolved in in isopropyl alcohol (1%) in n-hexane. For the reverse phase analysis of carotenoids (RP), the organic dry residue is dissolved in methanol:MTBE, 50:50 (v/v).

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Chromatographic determination of retinols, carotenoids and tocols

Extracted samples are analysed by high-performance liquid chromatography (HPLC). Under normal phase conditions, a 250 x 4.6 mm i.d., 5 mm particle size, Kromasil Phenomenex Si column (Torrance, CA) is used. A HPLC Dionex (Sunnyvale, CA) analytical system, consisting of a 50 µL injector loop (Rheodyne, Cotati) and a Ultimate 3000 (U3000) pump system is used. Spectrophotometric detection for carotenoids □□ = 450 nm) and fluorimetric detection for tocols (□exc = 290 nm, □em = 330 nm) and retinols (□exc = 325 nm, □em = 475 nm) are achieved by means of a UVD170 spectrophotometer and a RF 2000 spectrofluorimeter (Dionex, Sunnyvale), respectively. For tocol determination the mobile phase is n-hexane/ ethyl acetate/acetic acid (97.3:1.8:0.9 v/v/v) at a flow rate of 1.6 ml/min (Panfili et al., 2003). For carotenoid determination through normal phase HPLC (NP), the mobile phase is n-hexane/isopropyl alcohol (5%) at a flow rate of 1.5 ml/min (Panfili et al., 2004) or, for vegetables, a multilinear gradient elution from 10% (A) to 20% (B) of isopropyl alcohol, with a flow rate of 1.5 mL/min. Retinol separation is performed at a flow rate of 1.5 ml/min with isopropyl alcohol (1%) in n-hexane and n-hexane in a multi linear gradient, as described by Panfili et al. (1994). Carotenoids (carotenes) are also analyzed through a reverse phase HPLC method, at a flow rate of 1 mL/min, by using a 5 µm, C30 YMC (Hampsted, NC, USA) stainless steel column (250×4.6 mm i.d.). The mobile phase is methanol: MTBE: water. The gradient profile is given in Mouly et al, 1999. Tocol analysis is performed through a normal phase HPLC, as already reported for carotenoids and as Panfili et al., 2004.

Thiamine and riboflavin analysis

The extraction procedure of Hasselmann et al., 1989, is applied. A variable amount of sample is weighted in 100 mL volumetric flasks; 20 mL of 0.1 N HCl are added, followed by heating in a water bath at 100 °C for 30 min. Further details are reported in Niro et al., 2019. A HPLC Dionex (Sunnyvale, CA), with a U3000 pump and an injector loop (Rheodyne, Cotati), is used to separate the extracts, through a 5 µm C18 Luna Phenomenex stainless steel column (250×4.6 mm i.d.) (Torrance, CA, USA). The mobile phase iss methanol:sodium acetate (40:60 v/v), at a flow rate of 0.8 mL/min. Fluorometric detection is performed at a □exc of 453 nm and a □em of 580 nm, for riboflavin, and at a □exc of 366 nm and a □em of 453 nm, for thiamine, after its derivatization to thiocrome, by means of a Dionex RF 2000 spectrofluorimeter.

INTERPRETATION OF THE RESULTS:

A Dionex Chromeleon Version 6.6 chromatography system is used to process data. Compounds are identified by comparing their retention times with standard solutions and through their spectral characteristics, and quantified through the calibration curves of each standard solution. Vitamin E activity is expressed as Tocopherol Equivalent (T.E.) (mg/100 g), as in Sheppard et al.,1999. Vitamin A activity is expressed as Retinol Equivalent (R.E. mg/100 g) as in EFSA, 2015.

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DEVICE PERFORMANCE

Laboratory equipment is subjected to periodic maintenance by the dedicated technical assistance service.

LITERATURE:

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49. Official quality parameters of virgin olive oil: free acidity index cold method

EQUIPMENT AND REAGENTS:

Reagent and Materials	
Diethyl ether	Burette graduated in 0.05 mL
Ethanol 95 % (v/v)	Pipette
KOH 0.1 N	Analytical balance
Phenolphthalein (10 g/L in 95-96 % ethanol)	250 mL Erlenmeyer flask

METHOD DESCRIPTION:

This method describes the determination of free fatty acids in olive oils and olive pomace oils. The content of free fatty acids is expressed as acidity, calculated as the percentage of oleic acid. An olive oil sample is dissolved in a mixture of solvents and the free fatty acids present are titrated using a potassium hydroxide solution.
The weight of sample depends on expected acidity, as showed in Table 2.

Expected acidity (% oleic acid)	Mass sample (g)	Weighing accuracy (g)
0 to 2	10	0.02
Between 2 and 7.5	2.5	0.01
More than 7.5	0.5	0.001

Samples will be taken in 250 mL Erlenmeyer flask. Then, 50 mixture of diethyl ether: ethanol (1:1; v/v), previously neutralized with KOH solution and phenolphthalein solution, is added to dissolve the sample. Titrate while stirring with the 0.1 mol/L solution of potassium hydroxide until the indicator changes (the color of the colored indicator persists for at least 10 seconds).

INTERPRETATION OF THE RESULTS:

1. **General remarks**

This method shows the amount of free fatty acids presents in olive oil samples. It could be used as stability indicator because free fatty acids could be degraded faster. For olive oil, commercial classification is provided by International Olive Council (Table 3).

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Commercial Category	Free acidity (% oleic acid)
Extra Virgin Olive Oil (EVOO)	≤ 0.80
Virgin Olive Oil (VOO)	≤ 2.00
Ordinary Virgin Olive Oil (OVOO)	≤ 3.30
Lampante Virgin Olive Oil (LVOO)	> 3.30
Refined Olive Oil (ROO)	≤ 0.30
Olive Oil (ROO + VOO)	≤ 1.00
Crude Olive Pomace Oil (COPO)	No limit
Refined olive Pomace Oil (ROPO)	≤ 0.30
Olive Pomace Oil (ROPO + VOO)	≤ 1.00

2. **Methods of analysis**

Acidity, expressed as % of oleic acid, is obtained by equation 1

$$Free\ acidity\ (\%\ oleic\ acid) = \frac{V * c * M}{10 * m}\ \ (Equation\ 1)$$

where: **V** is volume of titrated potassium hydroxide solution used (mL); **c** is concentration of titrated potassium hydroxide solution (mol/L); **M** is molar mass of oleic acid (282 g/moL) and **m** is the mass of the sample, expressed in grams.

LITERATURE:

1. International Olive council. Determination of free fatty acids, cold method. Coi/t.20/doc. No 34/rev. 1. 2017.
2. International olive council. Trade standard on olive oils and olive-pomace oils. Coi/t.15/nc no 3/rev. 17. 2021.

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50. Official quality parameters of virgin olive oil: peroxide index

EQUIPMENT AND REAGENTS:

Reagent and Materials	
Chloroform	Burette graduated in 0.05 mL
Glacial acetic acid	Pipette
Potassium iodide saturated (1.4 g/ mL water at room temperature)	Analytical balance
Sodium thiosulphate (0.01 mol/L)	250 mL Erlenmeyer flask
Starch solution (10 g/L)	Analytical balance
Distilled water	

Table 1: Reagents and Materials to analyze peroxide index.

METHOD DESCRIPTION:

This method describes the determination of peroxide value in olive oils and olive pomace oils. The peroxide value is the quantity of those substances in the sample, expressed in terms of milliequivalents of active oxygen per kilogram, which oxidize potassium iodide under the operating conditions described. The weight of sample depends on expected peroxide value, as showed in Table 2.

Expected peroxide value (meq O_2/ Kg)	Mass sample (g)
0 to 12	5.0 to 2.0
12 to 20	2.0 to 1.2
20 to 30	1.2 to 0.8
30 to 50	0.8 to 0.5
50 to 90	0.5 to 0.3

Table 2: Relation between expected peroxide value and mass sample.

Samples will be taken in 250 mL Erlenmeyer flask, preferably filled with an inert and dried gas. Add 10 mL of chloroform and dissolve the sample, then add 15 mL of glacial acetic acid and 1 mL of potassium iodide, close the flask, shake for one minute and leave them five minutes in darkness. After that, add 75 mL of distilled water and titrate with thiosulphate solution, using starch solution as indicator.

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INTERPRETATION OF THE RESULTS:

1. **General remarks**

This method shows quantity of substances in the sample, expressed in terms of milliequivalents of active oxygen per kilogram, which oxidize potassium iodide and could be used as an indicator of initial degradation of oils. For olive oil, commercial classification is provided by International Olive Council (Table 3).

Commercial Category	Peroxide value (meq O_2/ Kg)
Extra Virgin Olive Oil (EVOO)	≤ 20.0
Virgin Olive Oil (VOO)	≤ 20.0
Ordinary Virgin Olive Oil (OVOO)	≤ 20.0
Lampante Virgin Olive Oil (LVOO)	No limit
Refined Olive Oil (ROO)	≤ 5.0
Olive Oil (ROO + VOO)	≤ 15.0
Crude Olive Pomace Oil (COPO)	No limit
Refined olive Pomace Oil (ROPO)	≤ 5.0
Olive Pomace Oil (ROPO + VOO)	≤ 15.0

Table 3: Peroxide values for olive oil commercial categories.

2. **Methods of analysis**

Acidity, expressed as % of oleic acid, is obtained by equation 2

$$Peroxide\ value\ (meq\ O_2/\ Kg) = \frac{V * T * 1000}{m}\ \ (Equation\ 2)$$

where: **V** is volume of titrated sodium thiosulphate solution used (mL); **T** is molarity of titrated sodium thiosulphate solution (mol/L), and **m** is the mass of the sample, expressed in grams.

LITERATURE:

1. International Olive Council. Determination of peroxide value. COI/T.20/Doc. No 35/Rev.1 2017.
2. International olive council. Trade standard on olive oils and olive-pomace oils. Coi/t.15/nc no 3/rev. 17. 2021.

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51. Official quality parameters of virgin olive oil: K values

EQUIPMENT AND REAGENTS:

Reagent and Materials	
Cyclohexane	Spectrophotometer in Ultra-Violet range
Iso-octane	One-mark 25 mL flask
	Analytical balance
	10 mm quartz cuvettes

Table 1: Reagents and Materials to analyze spectrophotometric values (K values)

METHOD DESCRIPTION:

Spectrophotometric examination in the ultraviolet can provide information on the quality of a fat, its state of preservation and changes brought about by technological processes. The absorption at the wavelengths specified in the method is due to the presence of conjugated diene and triene systems resulting from oxidation processes and/or refining practices. In this method, it is possible to use cyclohexane for measurements at 232, 266, 270 and 274 nm; or iso-octane for measurements at 232, 264, 268 and 272 nm. Samples will be taken in 25 mL flask, preferably filled with an inert and dried gas. To analyze K_{232} values, 0.05 g of sample will be taken in a 25 mL flask and will be filled with cyclohexane or iso-octane. To analyze K_{266}, K_{270} *and* K_{274} *or* K_{264}, K_{268} *and* K_{272}, 0.25 g of sample will be taken in a 25 mL flask and will be filled with cyclohexane or iso-octane, respectively.

INTERPRETATION OF THE RESULTS:

1. **General remarks**

This method can provide information on the quality, state of preservation and changes brought by technological process. For olive oil, commercial classification is provided by International Olive Council (Table 2).

Commercial Category	K_{232}	K_{270} or K_{268}	ΔK
Extra Virgin Olive Oil (EVOO)	≤ 2.50	≤ 0.22	≤ 0.01
Virgin Olive Oil (VOO)	≤ 2.60	≤ 0.25	≤ 0.01
Ordinary Virgin Olive Oil (OVOO)		≤ 0.30	≤ 0.01
Lampante Virgin Olive Oil (LVOO)			
Refined Olive Oil (ROO)		≤ 1.25	≤ 0.16
Olive Oil (ROO + VOO)		≤ 1.15	≤ 0.15
Crude Olive Pomace Oil (COPO)			
Refined olive Pomace Oil (ROPO)		≤ 2.00	≤ 0.20
Olive Pomace Oil (ROPO + VOO)		≤ 1.75	≤ 0.18

Table 2: Values for K_{232}, K_{270} or K_{268} and ΔK for olive oil commercial categories.

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2. **Methods of analysis**

The specific extinctions (extinction coefficients) at the various wavelengths calculated with equation 3

$$K_\lambda = \frac{E\lambda}{c * s} \quad (Equation\ 3)$$

where: **Kλ** is specific extinction at wavelength λ; **Eλ** is extinction measured at wavelength λ; **c** is concentration of solution (g/ 100 mL), and **s** is path length of quarts cuvette in cm.

The variation of the extinction (ΔK) is given by equation 4

$$\Delta K = K_m - (\frac{K_{m-4} + K_{m+4}}{2}) \quad (Equation\ 4)$$

where K_m is the specific extinction at the corresponding wavelength to each solvent used.

LITERATURE:

1. International Olive Council. Spectrophotometric investigation in the ultraviolet COI/T.20/Doc. No 19/Rev. 5. 2019.
2. International Olive Council. Trade standard on olive oils and olive-pomace oils. COI/T.15/NC No 3/Rev. 17. 2021.

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52. Particle size analysis

EQUIPMENT AND METHOD DESCRIPTION:

Mastersizer 2000 (MALVERN INSTRUMENTS)	
Task / Analysis	Determination of the particle size distribution of starch preparations using the laser diffraction technique
Research and analytical basis	Mie scattering theory
The basics of work	Development and implementation of measurement methods in accordance with the ISO 13320 standard
Equipment and general principle of operation	Applications: pharmacy, power industry, construction and mineral materials industry, food industry, scientific research. Measurement range from 0,02 to 2000 um thanks to the manufacturer's patented measurement technology using two laser beams (red laser L=633mm and blue laser L=466mm) Wet and dry method of measurement - in liquid and air dispersions Single-lens optical system Automatic alignment of the optical path and zeroing of the measurement system Standard SOP measurement procedures (Standard Operating Procedure) to ensure repeatability of measurement conditions Evaluation of the quality of the analysis results through the software Full automation of analyzer and attachment control

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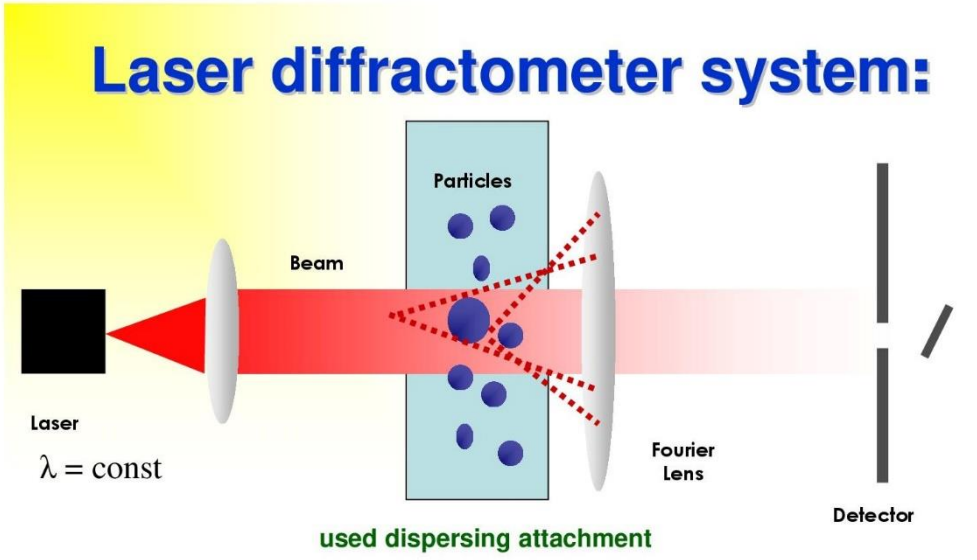

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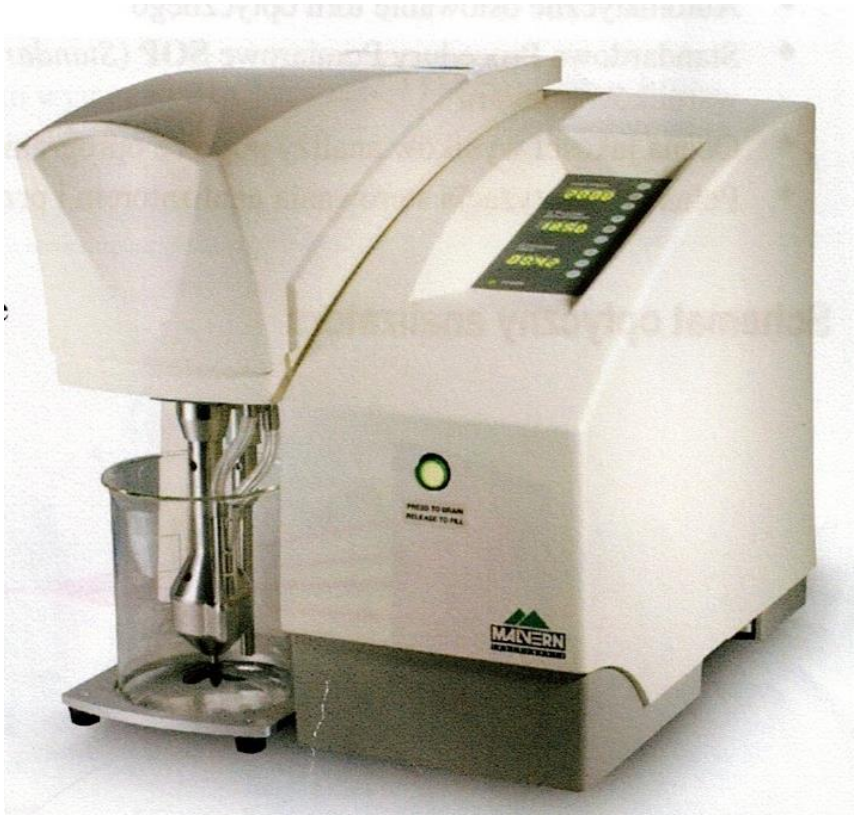
WET dispersion attachment Hydro MU (Mastersizer 2000)	
Task / Analysis	Semi-automatic high-volume attachment for measuring all materials with high density or complex granulometric distribution
Equipment and general principle of operation	measurement adapted to standard 600 and 1000 ml beakers the built-in ultrasonic probe supports the breaking of agglomerates lifting mechanism integrated pump head as well as the stirrer and ultrasonic probe facilitates the replacement of the dispersant liquid and the cleaning of the system The exceptionally efficient pump and agitator unit allows the dispersion of samples over a wide range of particle sizes Spill-resistant touch panel control control via SOP facilitates operation and ensures repeatable measurement conditions



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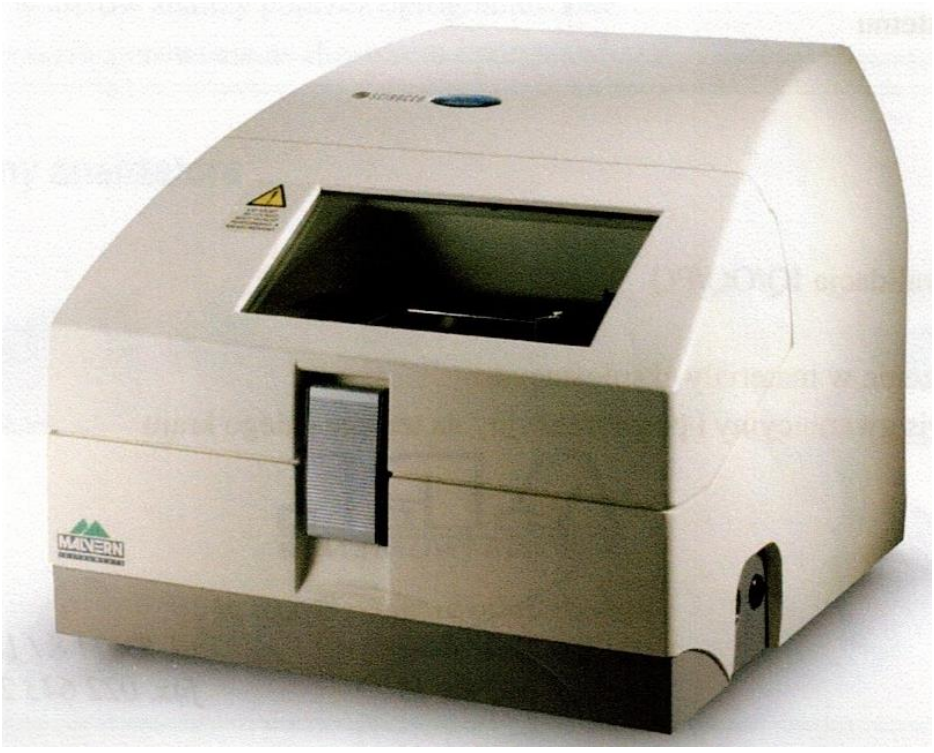
DRY dispersion attachment Scirocco 2000 (Mastersizer 2000)	
Task / Analysis	Accurate measurement of dry powders requires stringent control of dispersion conditions. The attachment disperses the particles by spraying them in the stream of compressed air. Precise control of dispensing and dispersion pressure ensures high reproducibility of measurement conditions in a wide range of samples, from brittle pharmaceuticals to shatter-resistant materials such as cement. Measurements are made in a sealed cell, which ensures safe handling of the material, especially when measuring pharmaceutical active substances, poisons or food additives
Equipment and general principle of operation	Automatic control via SOP of the stream of the sample fed and the pressure of the dispersing air allows the development of a measurement method in accordance with the ISO 13320 guidelines Dispersing air pressure in the range of 0 to 4 bar with a step of 0,1 bar and an accuracy of 0,02 A number of different trays feeding the sample to the attachment allows you to select the optimal amount of the analyzed substance in the range from a few mg to 200g Measuring cell with stable sample flow



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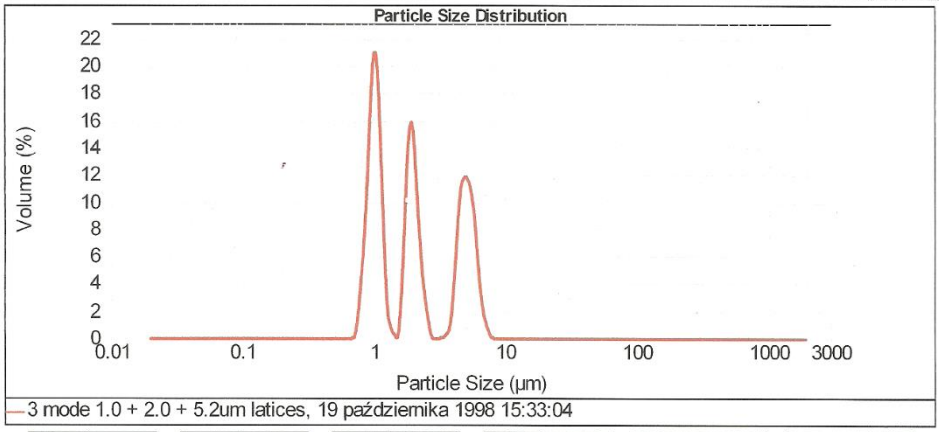
INTERPRETATION OF THE RESULTS:

MASTERSIZER 2000

Result Analysis Report

Sample Name: 3 mode 1.0 + 2.0 + 5.2um latices	**SOP Name:**	**Measured:** 19 października 1998 15:33:04	
Sample Source & type:	**Measured by:**	**Analysed:** 10 marca 1999 10:15:16	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Polystyrene latex	**Accessory Name:** Hydro 2000S (A)	**Analysis model:** Multiple narrow modes	**Sensitivity:** Enhanced
Particle RI: 1.590	**Absorption:** 0	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 8.22 %
Dispersant Name: Water	**Dispersant RI:** 1.330	**Weighted Residual:** 1.127 %	**Result Emulation:** Off
Concentration: 0.0015 %Vol	**Span :** 2.419	**Uniformity:** 0.756	**Result units:** Volume
Specific Surface Area: 3.52 m²/g	**Surface Weighted Mean D[3,2]:** 1.624 um	**Vol. Weighted Mean D[4,3]:** 2.553 um	

d(0.1): 0.934 um d(0.5): 1.863 um d(0.9): 5.441 um



Size (µm)	Volume In %
0.500	0.00
0.554	0.00
0.615	0.00
0.682	0.01
0.756	2.88
0.838	

Size (µm)	Volume In %
0.838	6.61
0.929	13.93
1.030	12.95
1.143	3.79
1.267	0.63
1.405	

Size (µm)	Volume In %
1.405	0.00
1.558	2.40
1.727	9.82
1.915	10.26
2.124	4.37
2.355	

Size (µm)	Volume In %
2.355	1.87
2.611	0.00
2.895	0.00
3.210	0.04
3.559	0.35
3.947	

Size (µm)	Volume In %
3.947	3.59
4.376	7.68
4.852	7.99
5.380	6.82
5.966	2.96
6.615	

Size (µm)	Volume In %
6.615	1.03
7.335	0.03
8.133	0.00
9.019	0.00
10.000	

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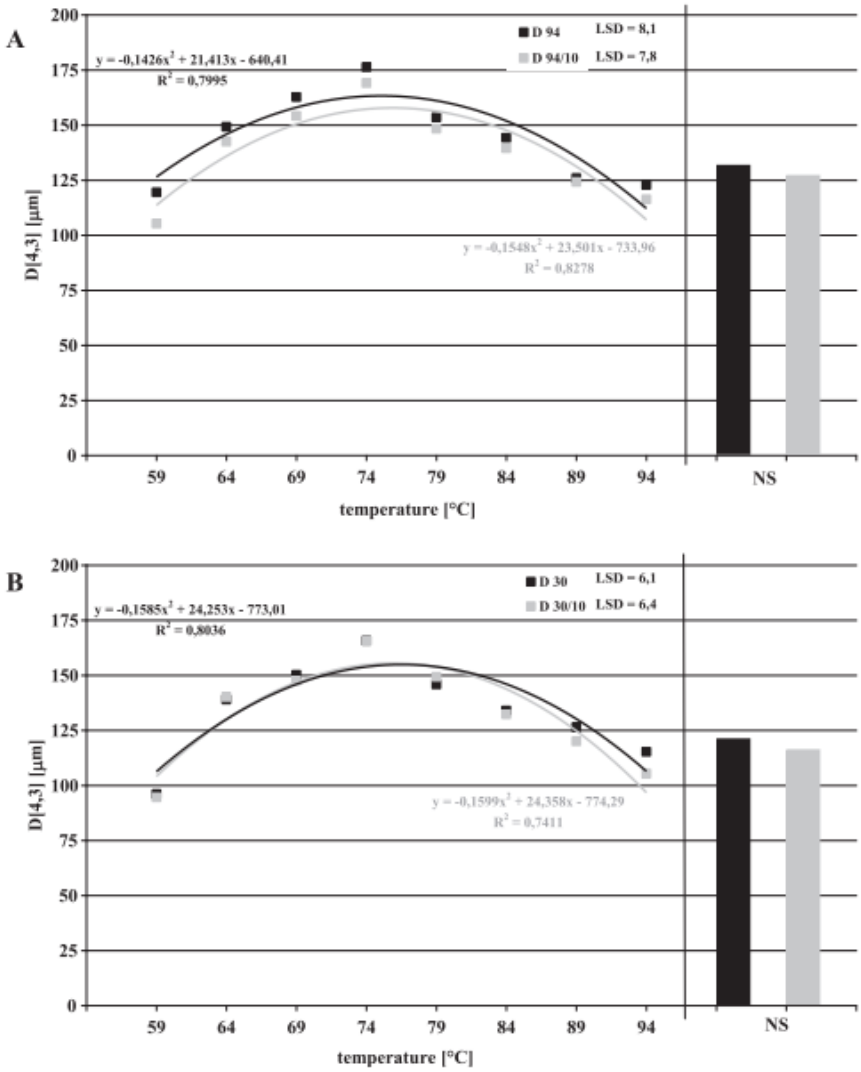

Fig. 5. Particle size in native starch (NS) paste and correlation between particle size in paste and temperature of starch dispersion heating before freezing: A – determined at 94 °C, B – determined at 30 °C.

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53. Pasta cooking quality—firmness and cooking loss

EQUIPMENT AND REAGENTS:

Equipment	
Equipment	Description
TAXT2 Texture Analyzer (Stable Micro Systems, Godalming, England)	Apparatus to evaluate pasta firmness
Hotplate	To bring to rolling boil water
Technical and analytical balances	Balances to weigh samples and reagents
Air oven	To Evaporate to dryness (constant weight)
Glassware and plastics of different capacities	
Reagent and Materials	
Distilled water; H_2SO_4 concentrated; Pure grade reagent $NaHCO_3$, Na_2CO_3, K_2SO_4, $MgCl_2$, $CaCl_2$ and NaOH from Sigma Chemical Co. (St. Louis, MO, USA).	

METHOD DESCRIPTION:

1. Texture Analyzer - Instrument setup *(1,2)*

1. Calibrate data-integration system with tooth in place. Use either 1 kg or full-load scale.
2. Set crosshead speed to 10.0 mm/min.
3. Position upper crosshead limit so that tooth clears sample by 1–3 mm.
4. Position lower crosshead limit so that tooth is 0.5 mm from bottom plate.
5. Set recorder speed at 100 mm/min.

2. Preparation of sample *(1)*

1. Place 300 ml distilled water into 500 mL beaker (one for each sample) and bring to rolling boil on hotplate. Composition of the cooking medium affects cooking loss. Substituting an artificial "hard water" medium for distilled water may better relate to many commercial water supplies. A suggested formula is: Into 16 liters distilled water, dissolve 7.68g Na_2CO_3, 9.60g $NaHCO_3$, 1.92g K_2SO_4, 1.92g $MgCl_2$, and 5.76g $CaCl_2$. A precipitate may form. Add about 5 mL 1:3 H_2SO_4/H_2O or a sufficient amount to clarify. Back titrate with 0.1N NaOH to pH 7.5. (Dilute this solution 8:1 with distilled water before using as cooking medium.)
Maintain one extra beaker of boiling distilled water for makeup purposes. Graduated beakers are useful for adjusting volume of cooking water during test.
2. Weigh two 25g portions of pasta for each sample being tested. Break pasta long goods or noodles into pieces approximately 5 cm long to permit free movement in boiling cooking medium.

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3. Cooking time *(1, 3)*

1. Determine cooking time for each product by adding one of 25g portions to beaker containing 300 mL boiling distilled water. Sample size of 25g is a suggestion only. Where material is limiting, smaller samples may be used. It is important to keep ratio of cooking water to sample to at least 10:1 to ensure return to rapid boil after addition of sample.
2. Start timer counting. Stir sample to make sure that pieces are separated.
3. Boiling stops when pasta is added. After cooking water has returned to rolling boil, maintain this condition throughout test. Partially cover beaker to help reduce evaporation and maintain consistent temperature. Use extra beaker of boiling water to maintain cooking water volume at least at 90% of original volume.
4. Remove piece from cooking water at 30 s intervals and squeeze it between two pieces of clear plastic. When center core just disappears, stop timer and record "cooking time."

4. Method - cooked firmness

1. Prepare samples for shear testing by cooking second portion of sample from step 2 of *Preparation of sample*. Use cooking technique described above, but do not remove any sample during cooking process.
2. When "cooking time" is reached, rapidly drain sample into Büchner funnel, retaining cooking water. Rinse sample with stream of distilled water for 30 s (approximately 50 mL distilled water) held at consistent temperature. Combine cooking and rinse waters for determining cooking loss.
3. Immediately transfer cooked samples into container of distilled water at room temperature.
4. Load sample onto bottom plate of Texture Analyzer, placing five strands of spaghetti (or equivalent width of other shapes) adjacent to one another.
5. Center sample under tooth, with axis of product at right angles to tooth. Compress to within 0.5 mm of base plate.
6. Return tooth to upper position, and wipe tooth and bottom plate clean before proceeding to next sample.
7. Repeat steps 4–6 at least twice more, using fresh subsamples.
8. Steps 4–7 should be performed at fixed times following completion of cooking to minimize changes resulting from storage in liquid medium. Cooked firmness of pasta and noodles is sensitive to time after cooking. Firmness determinations should be made at a set time after cooking. Recommended time for determination of firmness of first subsample is 10 min after cooking. Succeeding subsamples can be tested approximately 1 min apart, although time to prepare subsamples may vary with skill of operator.
9. Calculate maximum cutting stress by dividing maximum force value by tooth-sample contact area (in cm^2). Peak force is the maximum force achieved during cutting cycle, and work is represented by the area under curve. Mean values from results of all subsamples should be reported.

5. Method - Cooking loss

1. Pre-weigh 500 mL beaker to 0.01g.
2. Quantitatively transfer cooking/rinse water to beaker.

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3. Evaporate to dryness (constant weight) in air oven at 100 ± 1°C. Drying time is approximately 20hr but may vary with oven capacity, load, etc.
4. Cool beakers in desiccator and weigh to 0.01g. For 25g sample, increase in weight times 4 equals percent cooking loss.

DEVICE PERFORMANCE

Laboratory equipment is subjected to periodic maintenance by the dedicated technical assistance service.

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54. Positional analysis of fatty acids composition of egg-yolk phospholipids

EQUIPMENT AND REAGENTS:

Equipment	Description
Water bath	Adjustable to 100 °C
Rotary shaker	
Centrifuge	
Rotary vacuum evaporator	
SPE system	CHROMABOND SPE vacuum manifold for 12 positions and silica gel columns (Discovery DSC-Si SPE 500 mg)
GC	Agilent 6890N Network Gas Chromatograph with a flame ionization detector (FID)

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Column for GC	70% cyanopropyl polysilphenylene-siloxane column (TR FAME, 30 m × 0.25 mm × 0.25 µm)
Reagent and Materials	
chloroform, methanol, acetone, n-hexane	Pure p.a. from Chempur ® (Poland, Piekary Śląskie)
ethanol	Absolute pure from POCh (Poland, Gliwice)
Lipozyme® (immobilized lipase from Mucor miehei 86.8 U/g)	Fluka
48% BF_3/Et_2O	Fluka
anhydrous $MgSO_4$	POCh (Poland, Gliwice)
Fatty acids	Palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, arachidonic acid and docosahexaenoic acid, all analytical standard from Supelco

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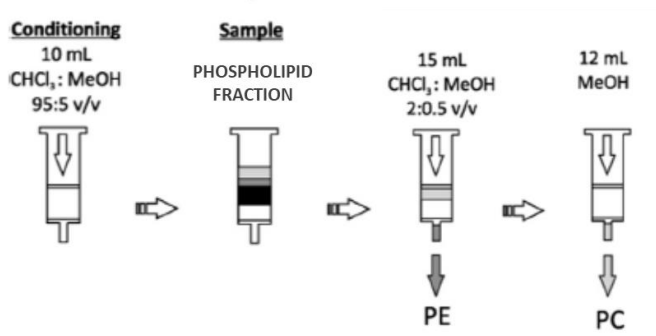
METHOD DESCRIPTION:

Phospholipids isolation

Phospholipids were isolated from egg yolk and purified according to a modified procedure described by Palacios and Wang (2005) but using methanol instead of ethanol for the extraction. 50 mL of methanol was added to the mixture of two fresh egg yolks (30 g) in a centrifuge bottle and stirred for 15 min. The mixture was then centrifuged and the supernatant containing water, polar lipids and some neutral lipids was filtered. The procedure was repeated three times. The methanol extracts were combined and the solvent was evaporated at 60 °C *in vacuo*. The remaining material was dissolved in 35 mL of hexane and transferred to a centrifuge bottle where 150 ml of cold acetone (−20 °C) was added and carefully stirred to precipitate PLs. The centrifuge bottle was then placed in an ice-water bath for 15 min and centrifuged (10,000g). The supernatant was removed and the precipitate was purified PLs.

Solid-phase extraction (SPE) – separation of phosphatidylcholine (PC) and phosphatidylethanolamine (PE)



SPE column was conditioned with 10 mL of chloroform:methanol (95:5, v/v), and then the phospholipid fraction (100 mg) dissolved in 1.0 mL of chloroform:methanol (95:5, v/v) was applied to the SPE cartridge. Then phospholipids were eluted with solvent mixtures of increasing polarity. First, PE was eluted with 15 mL of chloroform: methanol (2:0.5, v/v), and then 12 mL of methanol was used to elute PC. The fractions were evaporated using a rotary vacuum evaporator at 45 °C. The phosholipid fractions (PC and PE) purity was analyzed by thin-layer chromatography (TLC) (chloroform:methanol:water, 65:25:4,v/v/v).

Procedures for the analysis of fatty acids in *sn*-1 and *sn*-2 position of soy and egg yolk PLs

Position *sn*-1

2 mL of ethanol (95%) and Lipozyme (0.4 g) were added to dissolve PC or PE (0.05 g) and the mixture was shaken vigorously (150 RPM) for about 8 h and then the enzyme was filtered off. Ethanol was removed at 45 °C on a rotary evaporator in *vacuo*. The remaining material was

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suspended in 2 mL of water and vigorously mixed using a vortex mixer. The obtained suspension was transferred to a 15 mL vial and 3 mL of hexane was added carefully to extract fatty acids (FAs) and fatty acids ethyl esters (FAEEs) released from the *sn*-1 position of PC. The mixture was shaken on a laboratory shaker (shaker speed: 80 rpm). The two phases obtained after extraction were separated. The water fraction containing 2-acyl LPC or 2-acyl LPE was left for further analysis of fatty acids. The solvent was evaporated from hexane phase at 45 °C in *vacuo* and 2 mL of anhydrous ethanol and 1 mL of boron trifluoride etherate (48% BF_3/Et_2O) were added to the remaining material containing the FAEEs and FAs. The mixture was heated under reflux for 3 min to convert free fatty acids into ethyl esters. The progress of the esterification reaction was controlled by TLC (eluent:hexane:diethyl ether, 7:1 v/v). After cooling the mixture was extracted with 1 mL of hexane and the organic layer was washed with saturated 5 mL of NaCl solution. Hexane extract was dried over anhydrous magnesium sulfate and analyzed directly by gas chromatography.

Position *sn*-2

The water fraction with 2-acyl LPC (or 2-acyl LPE) was evaporated at 60 °C in vacuo. The remaining material was dissolved in 0.5 mL of hexane and transfer to a centrifuge bottle where cold acetone (−20 °C, 10 mL) was added to precipitate 2-acyl LPC. The precipitate was carefully washed 3 times with 10 mL portions of cold acetone and the solvent was removed by decantation each time. The mixture was then centrifuged (10,000g, 1 min). The supernatant was removed and 10 mg sample of pure 2-acyl LPC (purity was controlled by TLC hexane: diethyl ether, 7:1 v/v) was dissolved in 2 mL of 0.5 M ethanolic NaOH solution and heated under reflux for 2 min. Afterward, 1 mL of boron trifluoride etherate (48% BF_3/Et_2O) was added and the mixture was heated again under reflux for 3 min. FAEEs obtained were isolated from the reaction mixture as described before and analyzed by gas chromatography. In this way FAs composition in the *sn*-2 position of the PC was determined.

Synthesis of fatty acids ethyl esters (FAEEs) standards

Fatty Acids Ethyl Esters (FAEEs) standards were prepared by ethylation of fatty acids according to the procedure: 10 mg of fatty acids, 2 ml of ethanol and 1 ml of boron trifluoride etherate (48% BF_3) were combined and heated under reflux for 3 min. After cooling, the mixture was extracted with 1 mL of hexane and the organic layer was washed with 5 mL of saturated NaCl solution. Hexane extract was dried over anhydrous magnesium sulfate.

Gas chromatography

The fatty acid ethyl esters (FAEEs) were analyzed by gas chromatography (GC) on A Varian Chrompack CP-3380 apparatus with a flame ionization detector (FID). The separation was achieved using a 70% cyanopropyl polysilphenylene-siloxane column (TR FAME, 30 m × 0.25 mm × 0.25 µm). The oven temperature was 140 °C, held 3 min, rise to 220 °C at rate of 5 °C/min and then to 260 °C at rate 30 °C/min and held 3 min, while the injector temperature was 250 °C and the FID temperature was set at 280 °C. Hydrogen was used as the carrier gas.

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INTERPRETATION OF THE RESULTS:

General remarks

The procedure of structural analysis of PC is shown in Fig. 1. The very important advantage of this methodology is using one hydrolysis reaction. Only three ingredients are used: 95% ethanol, PC or PE and immobilized enzyme (*sn*-1,3 specific lipase from *Mucor miehei* – Lipozyme®). The complete conversion of PC to 2-acyl LPC takes place after 8 h.

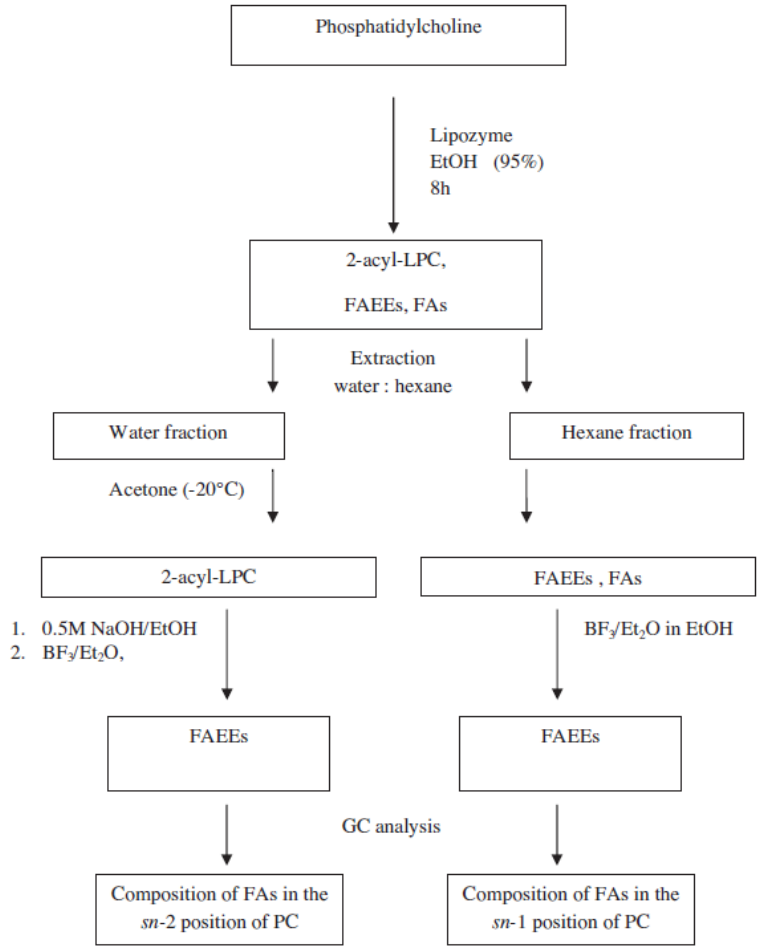

Fig 1.

The FAEEs obtained according to the above procedure are analyzed by gas chromatography The exemplary chromatograms are shown below.
FAs are identified by comparing the retention times of their ethyl esters with a standard FAEEs mixture.

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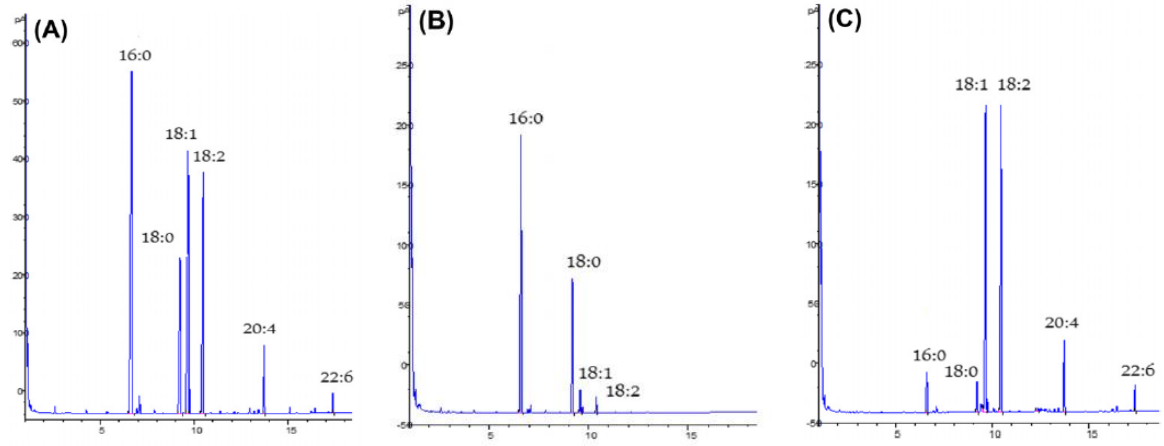

Fig 2. Chromatograms of ethyl esters of FAs in PC isolated from hen egg yolk. (A) PC (B) *sn*-1 position of PC (C) *sn*-2 position of PC.

RECOMMENDATIONS

- All the samples should be analyzed consecutively in the same day, for the same analyst to ensure repeatability
- In each case three replicates should be determined.

LITERATURE:

1. Palacios, L. E., Wang, T. 2005. Extraction of Egg-Yolk Lecithin. JAOCS, 82(8), p. 571–578.
2. Kiełbowicz G., Gładkowski, W., Chojnacka, A., Wawrzeńczyk, C. 2012. A simple method for positional analysis of phosphatidylcholine, Food Chemistry, 135, pp. 28–33. https://doi.org/10.1016/j.foodchem.2012.07.005.
3. Fauland, A., Trötzmüller, M., Eberl, A., Afiuni-Zadeh, S., Köfeler, H., Guo, X., Lankmayr, E. 2013. An improved SPE method for fractionation and identification of phospholipids, J. Sep. Sci. 36, pp. 744–751. DOI: 10.1002/jssc.201200708.

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55. Profile of organic acids

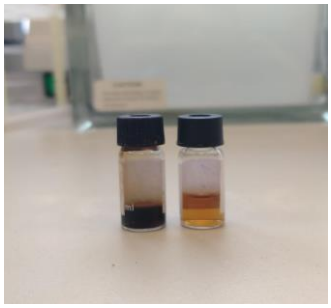
EQUIPMENT AND REAGENTS:

Equipment and reagents	
Equipment	Description
HPLC	Shimadzu Prominence –i LC – 2030C Plus
Column	ReproSil – Pur 120 C18-Aq, 250 mm x 4,6 mm; 4 µm
Automatic pipettes	Volumes : 1 – 10 µl, 20 – 200 µl, 100 – 1000 µl, 1 – 10 ml
Reagent and Materials	
Ultrapure deionized water	Milli-QR system
Disposable gloves	
Syringe filter	0,22 µm
H_2SO_4	pure for analysis
Acetic acid	Analytical standards
Formic acid	
Citric acid	
DL-Isocitric acid trisodium salt hydrate	
Maleic acid	
Malonic acid	
D-(+)- Malic acid	
Oxalic acid	
Succinic acid	
Shikimic acid	
D-(-) – Tartaric acid	

METHOD DESCRIPTION:

Dilution of fruit juices

100 µL of fruit juice is mixed with 900 µL of water in a chromatographic vial, which provides tenfold dilution of the sample. The photograph below represents the lemon juice concentrate (left) and diluted sample (right).



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External calibration

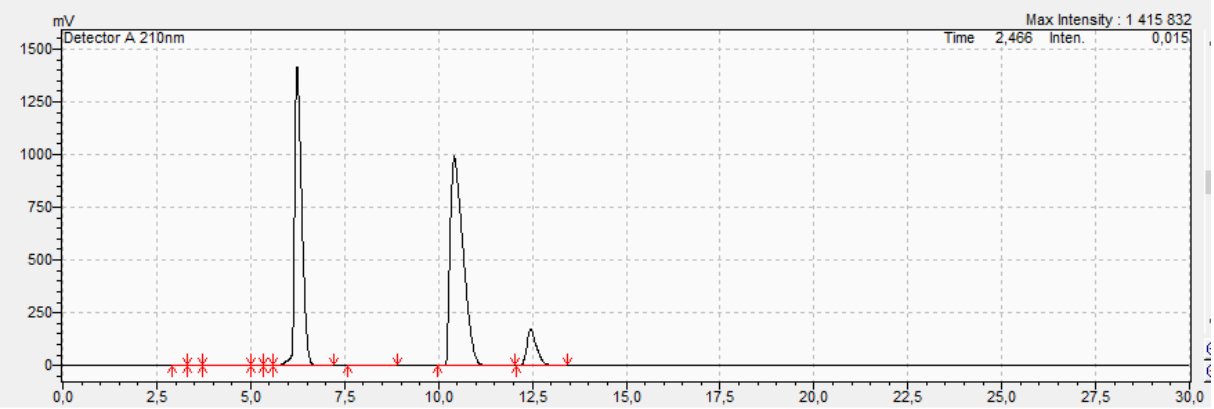
External calibration was accomplished using standards of 1 – 25 mg/L of each of the standards. Analysis was run in triplicate.

High-performance liquid chromatography (HPLC) conditions

Analysis are performed on Shimadzu Prominence - LC – 2030C Plus high performance liquid chromatograph with UV detection. Column used is ReproSil – Pur 120 C18-Aq (25 cm x 4,6 mm, 4 µm). Mobile phase consists of 0,01M H_2SO_4 with isocratic flow of 0,7 mL/min at 40 ˚C. UV wavelength is set to 210 nm, injection volume is 10 µl, and whole analysis is carried out in 40 minutes.

Interpretation of results

The following figure represents chromatogram obtained from measurements of sample containing 25 mg/ ml of malic acid (retention time of 6.2 minutes) and citric acid (retention time of 10.7 minutes) as an example:



For each concentration level (1, 5, 10, 15, 20, 25 mg/ml) of each analyte - mean peak area was calculated and used to create calibration curve presented below (Left - malic acid calibration curve; right - citric acid calibration curve).

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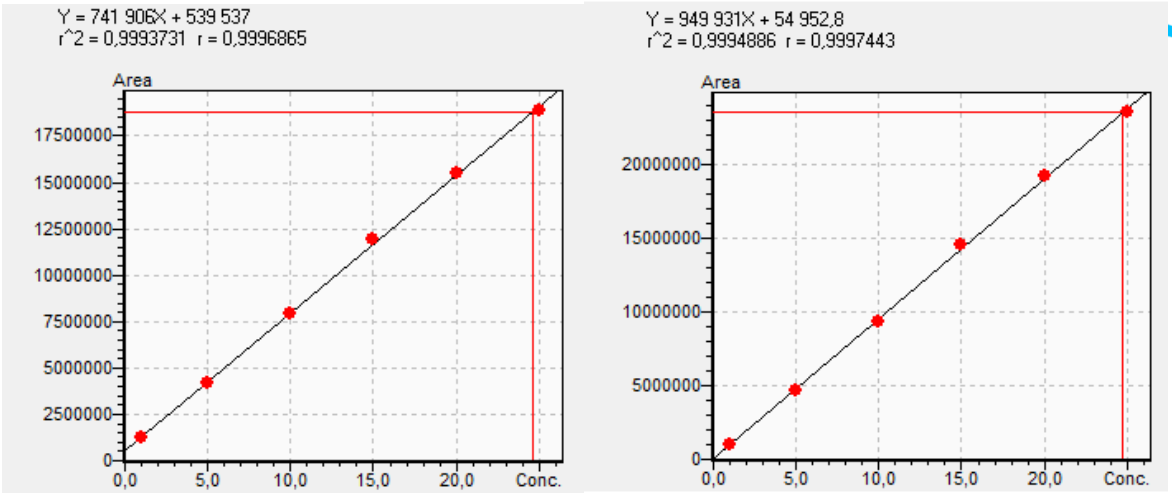

Recommendations:

Make sure that all glassware is as clean as possible and reagents used are of HPLC purity. Each analysis should be conducted in at least 3 repetitions. To prevent shifting of retention time make sure that mobile phase is at 2.1 pH.

LITERATURE:

1. Nour V., Trandafir I., Ionica M. E., 2010. HPLC Organic Acid Analysis in Different Citrus Juices under Reversed Phase Conditions" *Notulae Botanicae Horti Agrobotanici Cluj-Napoca,* 38 (1), pp. 44-48.
2. Lopez E. F., Gomez E. F., 1996. Simultaneous Determination of Major Organic Acids, Sugars, Glycerol, and Ethanol by HPLC in Grape Musts and White Wines. *Journal of Chromatographic Science*, 34.
3. Tasev K., Stefova M., Ivanova-Petropulos V. 2016. HPLC Method Validation and Application for Organic Acid Analysis in Wine After Solid-Phase Extraction" *Macedonian Journal of Chemistry and Chemical Engineering*, 35 (2), pp. 225-233.
4. Scherer R., Rybka A. C. P., Ballus C. A., Meinhart A. D., Filho J. T., Godoy H. T. 2012. Validation of a HPLC method for simultaneous determination of main organic acids in fruits and juices" *Food Chemistry* 135, pp. 150-154.

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56. Rapid procedure for amino acids analysis in foods

EQUIPMENT AND REAGENTS:

Equipment	
Equipment	Description
ICS6000 chromatographic system (Thermo Fisher Scientific S.p.A, Milano, Italy)	High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)
Microwave Digestion System (CEM Corporation, Matthews, NC)	Microwave digestion system designed for laboratory use
IKA A10 (IKAWERKE; GmbH &CO. KG, Staufen, Germany	Refrigerated laboratory mill for finely grinding samples before analysis
Rotary evaporator (BUCHI Italia S.r.l. Cornaredo, Italy)	Apparatus to evaporate samples to dryness
Glassware and plastics of different capacities	
Reagent and Materials	
Deionized water; HCl concentrated (37%); NaOH 50% (p/v) from Sigma Chemical Co. (St. Louis, MO, USA).; Amino acids standard from Sigma Chemical Co. (St. Louis, MO, USA).; Sodium Acetate anhydrous from Sigma Chemical Co. (St. Louis, MO, USA).	

METHOD DESCRIPTION:

Microwave protein hydrolysis *(1-3)*

The sample, corresponding to 25 mg of protein, is placed into four Teflon PFA digestion vessel of a microwave digestion system designed for laboratory use and 8 mL 6N HCl are added. Protein content is determined according AOAC method specific for each type of sample *(4).*

The vessel cup is screwed manually; the pressure and fibre optic probes are connected to the vessel with the triple ported cap. After the irradiation cycles (**Table 1**) the vessels are cooled and then removed. The hydrolysates are filtered by Whatman paper n.1. The filtered samples are evaporated to dryness by a rotary evaporator (bath temperature 40°C, pressure 25 mbar) and then re-dissolved with a precise volume of 0.1N HCl.

Before the analysis, samples are diluted 1:50 - 1:100 with ultra-pure water, filtered through 0.20 μm filter and then injected in the chromatographic system.

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Table 1. Operative conditions used for the microwave protein hydrolysis.

	1° CYCLE	2° CYCLE
Power (% 630 Watts)	85	85
Time (min)	1	5
Temperature (°C)	100	155
Pressure max (psi)	100	130

Chromatographic separation *(3,5)*

Separation is performed with an Aminopac PA10 analytical column 250x2 mm, with 8.5 µm particle size (Thermo Fisher Scientific). Quantitative determination is carried out at a flow rate of 0.25 mL/min using a mobile phase of water (Eluent E1), 250 mM sodium hydroxide (Eluent E2) and 1.0 M of sodium acetate (Eluent E3) as shown in **Table 2** and an optimized time-potential waveform as shown in **Table 3**.

Table 2. Gradient conditions for anion-exchange separation of amino acids.

Time (min)	**Eluent 1 Water (%)**	**Eluent 2 NaOH (%)**	**Eluent 3 Sodium acetate (%)**
0.0	80	20	0
2.0	80	20	0
12.0	80	20	0
16.0	68	32	0
24.0	36	24	40
40.0	36	24	40
40.1	20	80	0
42.1	20	80	0
42.2	80	20	0
62.0	80	20	0

Table 3. Integrated amperometry waveform used to detect amino acids**.**

Time (sec)	**Potential (V)**	**Integration**
0.00	+0.13	
0.04	+0.13	
0.05	+0.28	
0.11	+0.28	begin
0.12	+0.60	
0.41	+0.60	
0.42	+0.28	
0.56	+0.28	end
0.57	-1.67	
0.58	-1.67	
0.59	+093	
0.60	+0.13	

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INTERPRETATION OF THE RESULTS:

Amino acids identification and quantification is carried out by means of external amino acid standard.

Calculation of single amino acid content

Amino acid (g/100g) = ((SA x StdC)/StdA) x PM x D x 100/SW

where:

SA = Amino acid Sample Area

Std = Amino acid Standard Concentration

StdA = Amino acid Standard Area

PM = Amino acid Molecular Weight

D = dilution factor which consider the final volume of hydrolysate; the volume of hydrolysate dried by rotary evaporator; the volume of 0.1N HCl used to resume the sample after drying with rotary evaporator; the sample dilution (1:50 or 1:100) before the injection in the chromatographic system.

SW = sample weight.

DEVICE PERFORMANCE

Laboratory equipment is subjected to periodic maintenance by the dedicated technical assistance service.

LITERATURE:

1. Marconi E, Panfili G, Messia MC et al. 1996. Fast analysis of lysine in food using protein microwave hydrolysis and an electrochemical biosensor. Anal Letters 29 (7), pp. 1125-1137.
2. Marconi E, Panfili G, Bruschi L et al. 1995. Comparative study on microwave and conventional methods for protein hydrolysis in food. Amino Acids 8, pp. 201-208.
3. Messia MC, Di Falco T, Panfili G et al. 2008. Rapid determination of collagen in meat based foods by microwave hydrolysis and HPAEC-PAD analysis of 4-hydroxyproline. Meat Science 80, pp. 401-409.
4. AOAC. 2000. Official Methods of Analysis, 17th edn. Gaithersburg, MD; Association of Official Analytical Chemists.
5. Messia MC, Cuomo F, Falasca L et al. 2021. Nutritional and technological quality of high protein pasta. Foods, 10, pp. 589.

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57. SPME-ARROW as a facile tool for aroma analysis

EQUIPMENT AND REAGENTS:

Equipment and reagents - not supplied with the kit	
Equipment	Description
sq GC-MS Shimadzu 2020	for analysis of volatile compounds
Autosampler for Arrow	for analysis of volatile compounds
Helium for GC-MS	-
Arrows	Fibers are dependent on analyzed material
Optional equipment	
Software	Description
FFNSC 3 database	Database with aroma compounds
NIST20	Database of organic compounds
ACD Spectrus	Software for data handling
Reagent and Materials	
Internal standard	For quantification, depending on object
Authenticla samples of compounds	For quantification, depending on object
Samples of food, bevarages, biological materials etc.	

METHOD DESCRIPTION:

SPME Arrows takes to the next technological level by combining trace level sensitivity and high mechanical robustness with operational safety and full process tracebility. Solid Phase Microextraction is a solvent-free sample preparation technology. The arrows are either coated with a polymer, a sorbent or even both of them. This method will enable quantitative and qualitative analysis of VOCs in any food product as well as other biological samples. Volatile compounds are analyzed under head-space chromatographic conditions (approximately up to 20 carbons without oxygen atom and 15 with oxygen atom). Compared to the classical SPME technique, ARROW shows higher sensitivity and repeatability. Due to possibility SIM modes, it could be possible to quantify compounds on the ng/mL levels.

INTERPRETATION OF THE RESULTS:

1. **General remarks**

GC_MS equipped with SPME-ARROW allows to identify VOCs full profile in all foods, biological and environmental samples.

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2. **Methods of analysis**

Any food sample (liquid, e.g. alcohol, milk, beer, wine, etc.) or solid (sey fruit) is placed in a head-space vial together with an appropriately selected internal standard. After a 15-30 minute incubation time of the "AROOW" fiber, the volatile compounds are released in the chromatograph inlet. Identification of all volatile constituents obtained by HS-SPME analysis is based on comparison of experimentally obtained compound mass spectra with mass spectra available in NIST20, Massfinder FFNSC 3and databases. Also the experimentally obtained retention indeces (RI) by Kovats are compared with RI available in the NIST WebBook and literature data. The quantification analysis is performed using ACD/Spectrus Processor (Advanced Chemistry Development, Inc., Toronto, ON, Canada) through the integration of the peak area of the chromatograms.

DEVICE PERFORMANCE

Shimadzu 2020 equipped with SPME autosampler with single quadrupole detector. Available databases for qualitative analyses are: NIST20; FFNSC 3 database as well as Massfinder.

LITERATURE:

1. Łyczko, J., Masztalerz, K., Lipan, L., Iwiński, H., Lech, K., Carbonell-Barrachina, Á.A. and Szumny, A., 2021. Coriandrum sativum L.—Effect of Multiple Drying Techniques on Volatile and Sensory Profile. Foods, 10(2).
2. Kwaśnica, A., Pachura, N., Masztalerz, K., Figiel, A., Zimmer, A., Kupczyński, R., Wujcikowska, K., Carbonell-Barrachina, A.A., Szumny, A. and Różański, H., 2020. Volatile composition and sensory properties as quality attributes of fresh and dried hemp flowers (Cannabis sativa L.). Foods, 9(8), p.1118.
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58. Techniques for Antioxidant Activity and Total Phenolic Content evaluation in food

EQUIPMENT AND REAGENTS:

Antioxidant activity			TPC
ABTS$^{\bullet+}$	**DPPH$^{\bullet}$**	**FRAP**	
Equipments			
UV–visible spectrophotometer: (Helios Gamma model, UVG 1002E; Helios, Cambridge, UK)			
Ultrasonic water bath: (AU-65)			
Centrifuge			
Micropippets (range from2 µL to 1000 µL and to 1 mL to 10 mL)			
Reagents and materials			
Spectrophotometer cuvette (1.5 mL and 4.0 mL)			
Culture bottles (0.5 and 1 L)			
Plastic test tubes polypropylene (15x100mm - Capacity 12mL)			
Ultrapure water			
Methanol			
HCl			
Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)			Gallic acid
ABTS ([2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation)	DPPH (radical 2, 2-diphenyl-1-picrylhydrazyl)	TPTZ [2,4,6-Tris(2-pyridyl)-s-triazine]	Folin–Ciocâlteu
Potassium persulfate ($K_2S_2O_8$)		Sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$)	Sodium carbonate (Na_2CO_3)
		$FeCl3.6H_2O$	

METHOD DESCRIPTION:

The test consists of 4 main procedures, 3 for the antioxidant activity (AA) and 1 for the total phenolic content (TPC).

- ABTS$^{\bullet+}$ method
- DPPH$^{\bullet}$ method
- FRAP ferric reducing antioxidant power
- Folin–Ciocâlteu colorimetric method

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The extraction method is the same for the 4 procedures and consists of mixing the 0.5-1.0 g sample (preferably lyophilized) with 5-10 mL extractant [MeOH/water (80:20%, v/v) +1% HCl] depending on the matrix will be decided the sample amount and the extractant volume to be used. This mixture is sonicated at 20 °C for 15 min and left for 24 h at 4 °C. Then the extract is again sonicated for 15 min, and centrifuged at 15,000 g (or 10,000 rpm) for 10 min. The supernatant is collected in clean polypropylene test tubes and saved at 4 °C up to maximum 5 days.

$ABTS^{\bullet+}$ method

The ABTS [2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)] radical cation is used as described by Re et al. (1999).

This means that $ABTS^{\bullet+}$ reagent is prepared at 7 mM (10 mL) and $K_2S_2O_8$ at 2.45 mM (5 mL). Of the $K_2S_2O_8$ solution 1 mL is added to the 10 mL of ABTS•+ solution already prepared. This mixture is left for reaction at 20 °C for 16 h. The ABTS•+ solution is diluted in water (1 mL in 100 mL) until reaching an absorbance of 0.70 (± 0.02) with a wavelength of 734 nm.

Later, 10 µL of sample and 990 µL of $ABTS^{\bullet+}$ solution is placed in each cuvette with 15 seconds of difference among samples. After 6 minutes of reaction, the absorbance is measured.

The standard curve is made with Trolox from a concentration of 10 mM and the procedure is the same as for the samples. Depending on the plant material are we working with, the range of the curve is selected. It is proposed between 0.01–5.00 mM.

Reference

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med., 26(9-10), 1231-1237. doi:10.1016/s0891-5849(98)00315-3.

$DPPH^{\bullet}$ method

The free scavenging activity using the DPPH (radical 2,2-diphenyl-1-picrylhydrazyl) method was described by Brand-Williams et al. (1995).

For this DPPH• reagent is prepared at 100 µM with methanol, which needs to rest 30 min in the darkness for the reaction. Later the absorbance is measured at a wavelength of 515 nm. The absorbance must be less than 1.0. Finally, 10 µL of sample + 40 µL of methanol + 950 µL of DPPH solution are placed in each cuvette with 15 seconds of difference among samples. After 10 minutes of reaction, the absorbance is measured.

The standard curve is made with Trolox from a concentration of 10 mM and the procedure is the same as for the samples. Depending on the plant material we are working with, the range of the curve is selected. It is proposed between 0.01–5.00 mM.

DOI: https://doi.org/10.15414/2023.9788055226248

Reference

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. LWT - Food Sci. Technol., 28(1), 25-30. doi: 10.1016/S0023-6438(95)80008-5.

FRAP ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) is used as described by Benzie and Strain (1996). This method consists of the following steps:

- prepare the buffer solution of $C_2H_3NaO_2.3H_2O$ at pH = 3.6 (for a liter of buffer weigh 1.55 g of $C_2H_3NaO_2.3H_2O$ + 0.9 mL HCl).
- prepare 40 mM HCl
- prepare 10 mM TPTZ reagent with 40 mM HCl (For 10 mL, 31.23 mg of TPTZ is used with 10 mL of 40 mM) HCl
- prepare a solution 20 mM of $FeCl3.6H_2O$ (For 10 mL, 54.06 mg of $FeCl3.6H_2O$ is used with 10 mL of ultrapure water)

FRAP solution

- the reagents are prepared in a ratio 1: 1: 10 (TPTZ: FeCl3: Buffer)

Note: The reagent is light brown

Sample

- 10 µL of sample + 990 µL of FRAP solution are placed in each cuvette with 15 seconds of difference among samples. After 10 minutes of reaction, the absorbance is measured at 593 nm.

The standard curve is made with Trolox from a concentration of 10 mM and the procedure is the same as for the samples. Depending on the plant material with which you work, the range of the curve is selected. It is proposed between 0.01–5.00 mM. The absorbance reading should be between 0.2 and 0.8.

Reference

Benzie, I. F. F., & Strain, J. J. (1999) Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods in Enzymol., 299, 15-27. doi: 10.1016/s0076-6879(99)99005-5.

DOI: https://doi.org/10.15414/2023.9788055226248

Folin–Ciocâlteu colorimetric method

Total phenolic content (TPC) was quantified using Folin–Ciocâlteu colorimetric method described previously by Gao et al. (2000).

The methodology consists of the addition of 0.1 mL of sample + 0.2 mL of Folin–Ciocâlteu reagent + 2 mL of ultrapure water in cuvette of 4 mL. This mixture is rest in the darkness for 3 minutes. Then, 1 mL of 20% Na_2CO_3 is added and is let it to incubate at room temperature for 1 h. After the absorbance is measured at 765 nm in the UV visible spectrophotometer.
The standard curve is prepared with gallic acid from 1mM and prepared in the same way as the sample. The concentration proposed are 0, 25, 50 75, 100 and 125 µL.

Reference
Gao, X., Ohlander, M., Jeppsson, N., Bjork, L., & Trajkovski, V. (2000). Changes in antioxidant effects and their relationship to phytonutrients in fruits of sea buckthorn (*Hippophae rhamnoides L.*) during maturation. J. Agric. Food Chem., 48, 1485-1490. doi: 10.1021/jf991072g.

SPECTROPHOTOMETER DESCRIPTION:

Spectrophotometry is a basic and cheap technique to measure light absorption or the amount of chemicals in a solution. As observed in the followings figures the basics of the spectrophotometry are light source and color, because every chemical compound absorbs, transmits, and reflects light over an electromagnetic spectrum in wavelengths. For instance, when the light goes through any solution a part of it is absorbed, thus as the concentration of a substance increases light absorption also increases, and light transmission decreases. In this sense spectrophotometry is used in chemistry, biochemistry (for enzyme-catalysed reactions), physics, biology, and clinical studies.

The spectrophotometer measures the intensity of light of different wavelengths in the visible spectrum or the number of photons absorbed and is built as the name says from two instruments: a spectrometer and a photometer. The spectrometer produces the light of the wavelength, and the photometer measures the intensity of light by measuring the amount of light that passes through the sample. Spectrophotometers consist of a light source, a monochromator (which separates the polychromatic radiation of the light source into all its wavelength), a sample chamber containing a cuvette, a detector (such as a photomultiplier tube or photodiode) to detect the transmitted light, a digital display and a data analysis software package.

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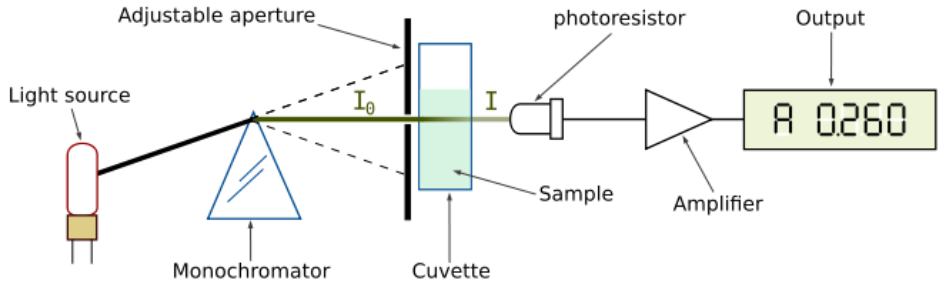

Source of the image: https://www.mrclab.com/spectrophotometers

There are two types: a single beam, and double beam. Single beam spectrophotometers are generally more compact and the optics in a double beam can permit higher levels of automation, better precision and can correct for background absorption of the solvent. With the double beam spectrophotometer, one beam passes through the sample, and the other through a reference solution or the solvent.

Source of the image: personal archive

Different compounds absorb best at different wavelengths. A UV-visible spectrophotometer uses light over the ultraviolet range (185 – 400 nm) and visible range (400 – 700 nm) of the electromagnetic radiation spectrum. Whereas an IR spectrophotometer uses light over the infrared range (700 – 15000 nm).

Source of image: https://tipbiosystems.com/blog/spectrophotometry/

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Ultraviolet (UV) and visible (VIS) spectroscopy show electronic transitions in atoms and molecules, to measure this a spectrophotometer is used. Compounds that absorb in the visible region are colored, whereas ones that absorb only in the UV region are colorless. UV-VIS spectrophotometer usually uses two light sources. A deuterium lamp is used for the UV region and a tungsten lamp for the VIS region. These lights reach the monochromator via a mirror. The wavelength for red light is between 700 and 750 nm and blue between 400 and 450 nm. If the wavelength is shorter than 350 nm it is UV and has more energy.

INTERPRETATION OF THE RESULTS:

3. **General remarks**

The radical $ABTS^{+}$, belongs to its acid precursor 2,2'-azinobis- (3- ethylbenzothiazoline) -6-sulfonic (ABTS), is has a bluish-green color, with a visible absorption spectrum, which is measured in the spectrophotometer at 734 nm wavelength (Re et al., 1999). The generation of the radical can be carried out enzymatically (myoglobin or horseradish peroxidase), chemically (peroxyl radical, potassium persulfate or manganese dioxide) and electrochemically.

The DPPH radical (2,2-diphenyl-1-picrilhydrazil radical) is purple in color, with an absorption spectrum at 515 nm and reacts with the antioxidant, decreasing its absorbance value in order to determine the degree to which it is captured. This is a direct determination (Brand-Williams et al., 1999).

The FRAP method consists of a reduction of the ferric tipyridyltriazine complex (TPTZ) reduction to the ferrous complex with an antioxidant and in an acid medium. This is an indirect determination as the absorbance increases as the TPTZ complex is reduced. It is a reaction that is measured at a wavelength of 593 nm (Benzie and Strain, 1996).

The measurement of the content of total phenolic content using the Folin–Ciocâlteu colorimetric method consists of measuring the color change from yellow to blue that accompanies the reaction of phenolic compounds with the mixture of sodium tungstate and sodium molybdate in phosphoric acid. This color change is measured spectrophotometrically at wavelength 765 nm, so that the blue coloration increases with increasing phenol concentration.

4. **Methods of analysis**

The antioxidant activity and the total phenolic content are obtained from the absorbance given by the spectrophotometer and using the sample weight, extraction volume, and the calibration curve. In the below section is showed an example of the calibration curve, an excel table with the data and the equations used to calculate the antioxidant activity and the total phenolic content:

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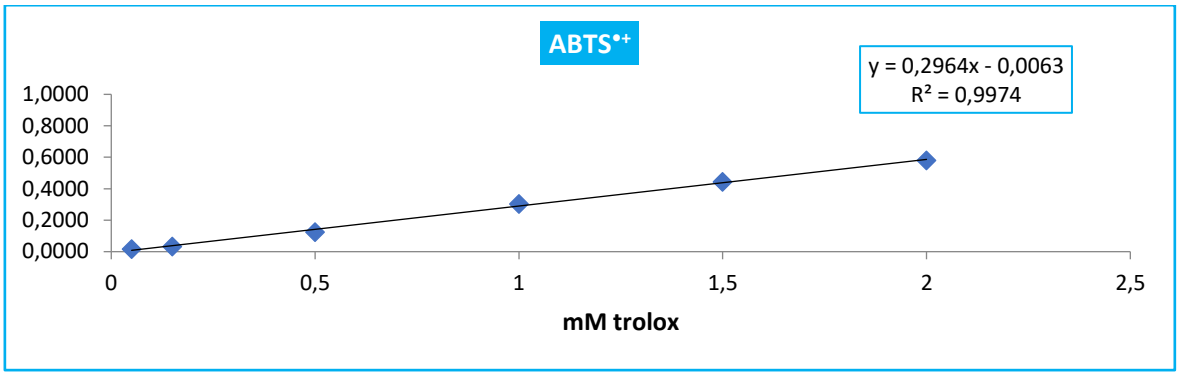
ABTS$^{\bullet+}$



Sample	Initial ABS	Spectro-photometer ABS	AA (calculation)	Weight (g)	Extractant volume (mL)	mmol/ g dry weight	Dilution	mM Trolox dm
Almond	0.707	0.686	0.09	0.4914	10	1.87	1	1.87
Almond	0.707	0.686	0.09	0.4961	10	1.86	1	1.86
Almond	0.707	0.684	0.10	0.5103	10	1.94	1	1.94
AlmECO	0.707	0.674	0.13	0.5083	10	2.61	1	2.61
AlmECO	0.707	0.669	0.15	0.4979	10	3.00	1	3.00
AlmECO	0.707	0.670	0.15	0.4991	10	2.93	1	2.93

$$AA_{calculation} = \frac{(Initial\ absorbance\ -\ Spectrophotometer\ absorbance)\ +\ 0.0063}{0.2964}$$

$$AA_{mmol/g} = \frac{(AAcalculation \times Extractant\ volume)}{weight}$$

This is an example of the antioxidant activity and total phenolic content determinations on samples containing almonds cultivated under conventional and ecological conditions. According to these results the AlmECO had a higher antioxidant activity than those almonds cultivated under conventional conditions.

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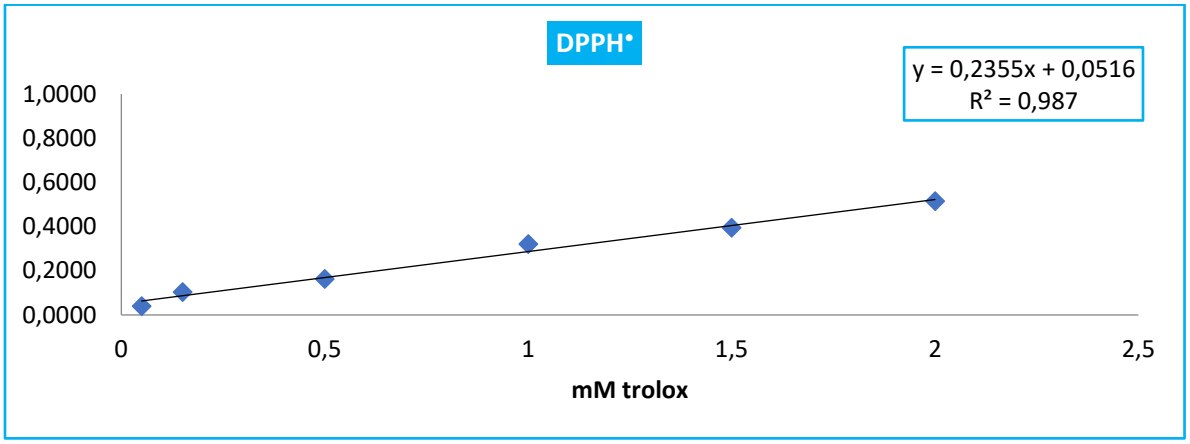
DPPH•



Sample	Initial ABS	Spectro-photometer ABS	AA (calculation)	Weight (g)	Extractant volume (mL)	mmol/ g dry weight	Dilution	mM Trolox dm
Almond	0.989	0.390	2.32	0.4914	10	47.3	1	47.3
Almond	0.989	0.396	2.30	0.4961	10	46.3	1	46.3
Almond	0.989	0.378	2.38	0.5103	10	46.5	1	46.5
AlmECO	0.989	0.314	2.65	0.5083	10	52.1	1	52.1
AlmECO	0.989	0.325	2.60	0.4979	10	52.2	1	52.2
AlmECO	0.989	0.319	2.63	0.4991	10	52.6	1	52.6

$$AA_{calculation} = \frac{(Initial\ absorbance\ -\ Spectrophotometer\ absorbance)\ -\ 0.0516}{0.2355}$$

$$AA_{mmol/g} = \frac{(AAcalculation \times Extractant\ volume)}{weight}$$

Same as in the first antioxidant activity method, the spectrophotometer absorbance is rested from the reagent absorbance and the graphical representation helps to obtain the equation needed to calculate the concentration of the samples. In the DPPH• method, it can be observed how lower values of absorbance reach to the higher values of absorbance. Here also, is showed how the ecological system might increase the antioxidant activity of the almonds.

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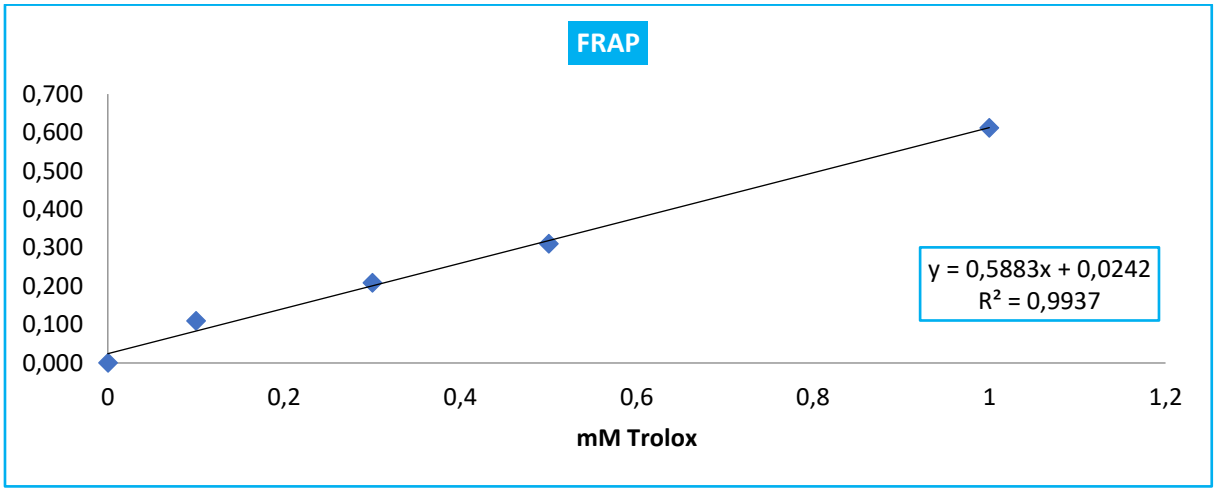
FRAP



Sample	Spectro-photometer ABS	AA (calculation)	Weight (g)	Extractant volume (mL)	mmol/ g dry weight	Dilution	mM Trolox dm
Almond	0.107	0.14	0.4914	10	2.86	1	2.86
Almond	0.105	0.14	0.4961	10	2.77	1	2.77
Almond	0.107	0.14	0.5103	10	2.76	1	2.76
AlmECO	0.130	0.18	0.4973	10	3.62	1	3.62
AlmECO	0.145	0.21	0.5015	10	4.09	1	4.09
AlmECO	0.140	0.20	0.5003	10	3.93	1	3.93

$$AA_{calculation} = \frac{(Spectrophotometer\ absorbance\ -\ 0.0242)}{0.5883}$$

$$AA_{mmol/g} = \frac{(AAcalculation \times Extractant\ volume)}{weight}$$

In the FRAP method, the highest the absorbance value the higher the antioxidant activity. As observed, FRAP method also shows that the AlmECO presented a higher antioxidant activity than conventional almonds.

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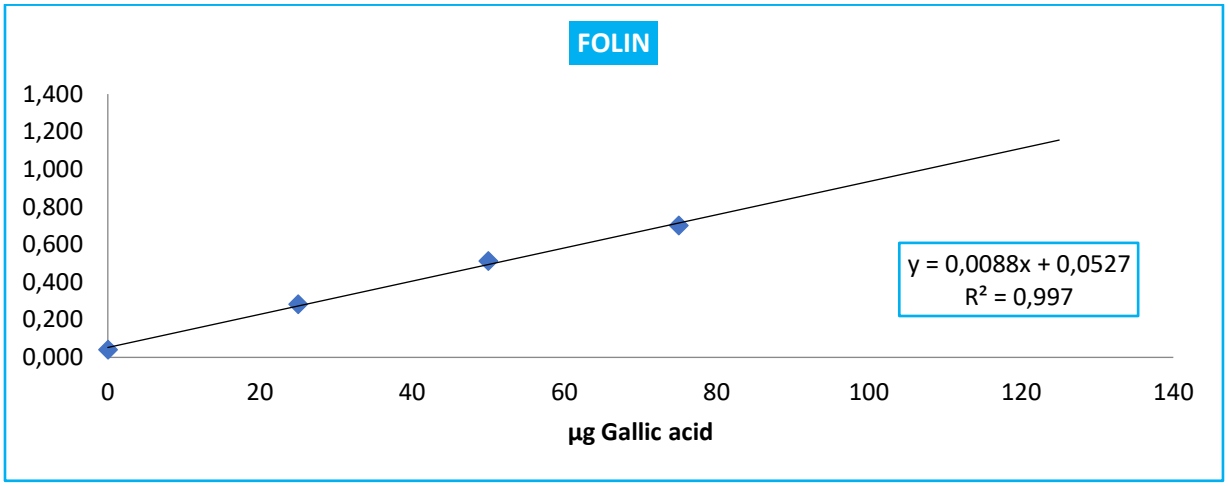
Folin–Ciocâlteu



Sample	Extractant volume (mL)	Sample volume (mL)	Weight (g)	Dilution	Absorbance	TPC (mg/100 g)
Almond	10	0.1	0.4914	1	0.073	46.9
Almond	10	0.1	0.4961	1	0.076	53.4
Almond	10	0.1	0.5103	1	0.080	60.8
AlmECO	10	0.1	0.4973	1	0.087	78.4
AlmECO	10	0.1	0.5015	1	0.086	75.5
AlmECO	10	0.1	0.5003	1	0.086	75.6

$$TPC_{mg/100\,g} = \frac{(Absorbance\ -\ 0.0527) \times (Extractant\ volume \times 100 \times Dilution\ factor)}{0.0088 \times \text{Sample volume} \times 1000 \times \text{weight}}$$

Finally, the higher values of antioxidant activity in AlmECO are also corroborated by the higher total phenolic content values for the same samples.

DEVICE PERFORMANCE

All evaluation experiments with respect to the assay specificity and sensitivity have been performed with vegetables extracts.

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Lipan, Leontina, Aarón A. Carbonell-Pedro, Belén Cárceles Rodríguez, Víctor Hugo Durán-Zuazo, Dionisio Franco Tarifa, Iván Francisco García-Tejero, Baltasar Gálvez Ruiz, Simón Cuadros Tavira, Raquel Muelas, Esther Sendra, Ángel A. Carbonell-Barrachina, and Francisca Hernández. 2021. "Can Sustained Deficit Irrigation Save Water and Meet the Quality Characteristics of Mango?" Agriculture 11, no. 5: 448. https://doi.org/10.3390/agriculture11050448

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Sánchez-Rodríguez, Lucía, Marina Cano-Lamadrid, Ángel A. Carbonell-Barrachina, Aneta Wojdyło, Esther Sendra, and Francisca Hernández. (2019). Polyphenol Profile in Manzanilla Table Olives As Affected by Water Deficit during Specific Phenological Stages and Spanish-Style Processing. Journal of Agricultural and Food Chemistry 67 (2), 661-670. DOI: 10.1021/acs.jafc.8b06392

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Universidad Miguel Hernández de Elche, Spain
Prof. Francisca Hernández García
Leontina Lipan, Junior researcher

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59. Texture in yoghurt

EQUIPMENT AND REAGENTS:

Equipment and reagents - not supplied with the kit	
Texture Analyser TA-XT2	Stable Micro Systems, Surrey, England Stable Micro Systems, Surrey, England
Cylindrical probe 10 mm diameter	P10

METHOD DESCRIPTION:

Penetration test

Penetration test is performed with a Texture Analyser TA-XT2 (Stable Micro Systems, Surrey, England) with a 5 kg load cell. Constant speed penetration tests is performed directly on cylindrical containers (4.5 cm diamete, 4 cm height). The measure of the sample takes place in the cylindrical containers in which yogurts' fermentation took place. All instrumental texture analyses are conducted at 7-8ºC and spontaneuos syneresis was removed previously. This is a “destructive” test as no structure recovery is allowed. A cylindrical probe 10 mm diameter ebonite (P-10) will be introduced 15 mm into the samples at a speed of 1 mm s^{-1}. Triplicate measures for each yoghurt will be performed.

INTERPRETATION OF THE RESULTS:

General remarks

The units of the graph can be grams force or Newton force; the first maximum peak corresponds to the fracture point (the point at which the probe exceeds the resistance of the surface) (Force-versus-distance graphs) (Figure 1). From the force-versus-time curves, values for the maximum force (N) could be calculated as force at a distance of 15 mm (Fmax). The average, the standard desviation, the standard error and the coefficient of variation should be calculated to ensure good results.

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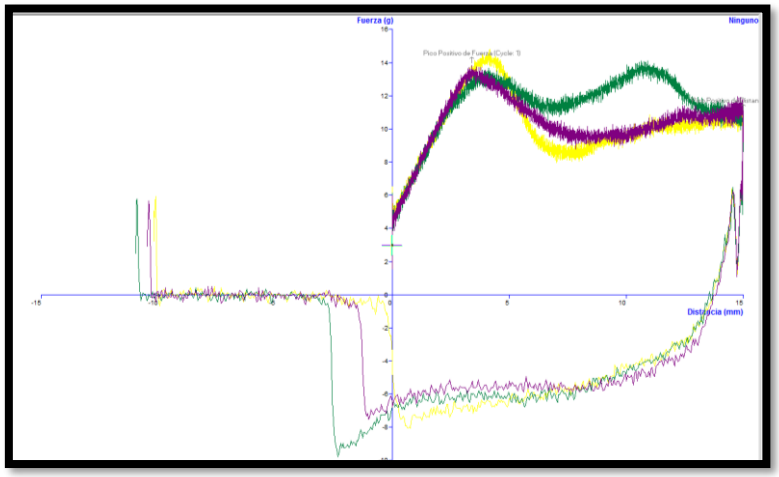

Figure 1. Force-versus-distance graph of yoghurt.

Table 1. Fmax (g) values of yoghurt.

	Fmax (g)
Replicate 1	13.9
Replicate 2	14.9
Replicate 3	14.2
Average	14.3
Standard desviation	0.5
Standard error	0.3
Coefficient of variation	3.5

RECOMMENDATIONS:

Force and distance must be calibrated before taking sample measurements.

This is just an example of application, methods can be developed for any food (fruits, meat fish, dairy products, spreads…), and calculations made by a macro designed for the required information from the sample.

LITERATURE:

Trigueros L, Pérez-Alvarez JA, Viuda-Martos M, Sendra, E. 2011. Production of low-fat yogurt with quince (*Cydonia oblonga Mill.)* scalding water. LWT - Food Science and Technology,44 (6), pp. 1388-1395, doi:10.1016/j.lwt.2011.01.012

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60. Volatile profile analysis by GCMS/MS

EQUIPMENT AND REAGENTS:

Equipment and reagents	
Equipment	Description
Chromatograph	GCMS/MS TQ 8040NX (Figure 1)
Oven columns	30-450 °C
Injection ports	1 (split-splitles)
Column	SLB-5ms; Suprawax-280
Autosampler	AOC-6000 plus
RTC LIQUID HS	Injection module for liquid samples with capacity up to 162 vials (2 mL)
HS KIT FOR GC2030	Automatic extraction module for head space (up to 45 vials)
SPME KIT	Automatic extraction module using chromatographic fibers (up to 45 vials)
FiberCondStation	Conditioning module for chromatographic fibers (Max. Temp. 300 °C)
Wash station	4 vials to washing solutions
Vortex	Shaker / oven (0-1000 rpm; 30-350 °C)
Optional equipment	
Equipment	Description
Library	NIST Mass Spectral Library 2017
Library	Mass Spectra of Flavors and Fragrances of Natural and Synthetic Compounds
Reagent and Materials	
Milli-Q Water	
Microextraction fiber (PDMS)	
Microextraction fiber (DVB/WR/PDMS)	
Microextraction fiber (DVB/PDMS)	
Microextraction fiber (WR/PDMS)	
Milli-Q Water	
n-alkane mixtures	
Pure compounds (internal standards)	
Filter tips	
Vials: 2 mL (IL) and 20 mL (HS & SPME)	

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Figure 1. Chromatograph GCMS/MS TQ 8040NX

METHOD DESCRIPTION:

In gas chromatography the sample is volatilized and injected at the top of a chromatographic column. Elution occurs by the flow of a mobile phase from an inert gas (He), and unlike most types of chromatography, the mobile phase does not interact with analyte molecules; its only function is to transport the analyte through the column.

The method consists of two differentiated processes: extraction of compounds from the food matrix and analysis of them (separation, identification, and quantification).

Extraction

To analyze the volatile compounds, present in the sample, a prior extraction of these is necessary. This will depend on the nature of the sample and the purpose of the study. Three types of extraction (Figure 2) can be performed with this equipment: liquid injection (IL), head space (HS) and solid phase micro extraction (SPME).

- IL: the sample must be extracted in an organic solvent. The equipment will take a defined volume of sample (1-10 µL) and inject it directly into the chromatograph.
- HS: the sample will be subjected to a heating and shaking process (previously defined by the user) after which, by means of a syringe (up to 2.5 mL), part of the headspace of the vial will be extracted and it will be injected directly into the chromatograph.

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- SPME: the sample will be subjected to defined temperature and agitation (up to 250 °C). During the process, or at the end, a chromatographic fiber will be exposed in the headspace of the vial. In this, the aromatic compounds will be adsorbed and subsequently desorbed in the injector of the chromatograph.

Figure 2. Methods of extraction of volatile compounds

Chromatographic analysis

The chromatographic program to be used will depend on the nature of the sample to be analyzed and the purpose of the study: study of target compounds or study of the profile of total volatile compounds. For the first case, the chromatographic program usually lasts short time, around 15 min, while in the second case, each sample can take up to 1 h.

For each type of sample, it will be necessary to set up the method (time, temperature, and column flow), by using samples of a similar nature to the test sample (if possible) or problem sample in greater quantity.

Once the peaks are separated, the software will identify the compounds based on the detection and quantification of the mass fragments. Based on the mass spectral bibliographies, it is possible to identify each of the compounds present and, by using internal standards, it is possible to perform semi-quantifications of compounds. If necessary, it is possible to create concentration curves, by using pure compounds, to obtain true and precise concentration values in samples.

Gas chromatography has limitations in three cases:

- low volatile compounds, generally those with a molecular weight greater than 350 a.m.u.
- compounds sensitive to even moderate temperature rise (certain compounds of biological interest)
- compounds that are in ionic form (since they are generally not very volatile)

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INTERPRETATION OF THE RESULTS:

This technique allows to separate, identify, and quantify the volatile compounds present in a food sample. The separation is done by the polarity and size of the compounds while the identification is made based on the fragmentation pattern of the molecules. The obtained result, chromatogram (Figure 3), shows the peaks as a function of the elution time (x-axis) and the intensity at which they have been detected (y-axis). In addition, for each of the peaks the information on the fragmentation of the masses is shown.

The results obtained can be compared with those in the bibliography, even though they have been obtained with different equipment, columns, times, etc., since they all work with the same electronic impact (70 eV).

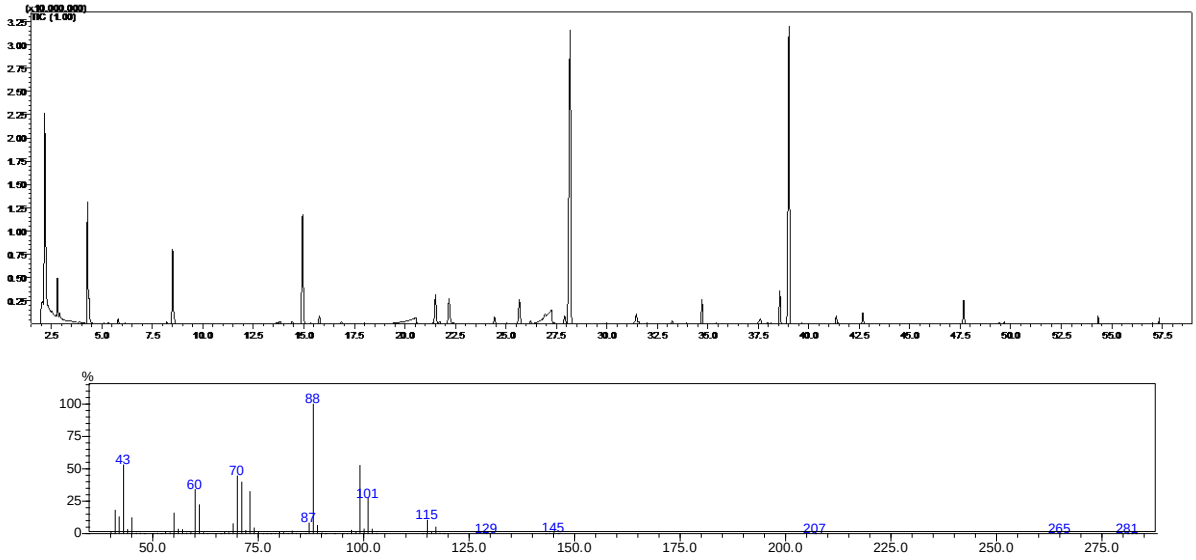

Figure 3. Example of chromatogram and mass spectra

EXAMPLES METHODS:

Below, you can see some methods, by way of example, in which the different conditions chosen for different food matrices are shown:

DOI: https://doi.org/10.15414/2023.9788055226248

WINE [1]	
Extraction	
Sample	5 mL wine + 10 mL water
Equilibration time	15 min
Equilibration temperature	40 °C
Extraction method	SPME; DVB/CAR/PDMS
Exposition time	50 min
Exposition temperature	40 °C
Chromatographic conditions	
Temperature injector	230 °C
Temperature detector	280 °C
Column	DB-5; 30 m × 0.25 mm ID × 0.25 µm
Column flow	(He) 1.0 ml/min
Split	1:10
Oven temperature program	50 °C; 2 °C/min up to 100 °C; 3 °C/min up to 180 °C; 20 °C/min up to 230 °C and hold 5 min

AROMATIC HERBS [2]	
Extraction	
Sample	15 g + 150 mL water (hydrodistillation)
Injection mode	IL
Chromatographic conditions	
Temperature injector	230 °C
Temperature detector	300 °C
Column	TRACSIL Meta X5; 30 m × 0.25 mm ID × 0.25 µm
Column flow	(He) 0.6 ml/min
Split	1:11
Oven temperature program	80 °C; 3 °C/min up to 210 °C, hold 1 min; 25 °C/min up to 300 °C, hold 3 min

CHOCOLATE [3]	
Extraction	
Sample	1 g + 15 mL water
Equilibration time	15 min
Equilibration temperature	50 °C
Extraction method	SPME; DVB/CAR/PDMS
Exposition time	55 min
Exposition temperature	50 °C
Chromatographic conditions	
Temperature injector	230 °C
Temperature detector	300 °C

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Column	DB-5; 30 m × 0.25 mm ID × 0.25 µm
Column flow	(He) 0.7 ml/min
Splitless	
Oven temperature program	40 °C; 2 °C/min up to 180 °C; 25 °C/min up to 300 °C and hold 1 min

PISTACHIO [4]	
Extraction	
Sample	1 g (grounded)
Equilibration time	5 min
Equilibration temperature	45 °C
Extraction method	SPME; DVB/CAR/PDMS
Exposition time	25 min
Exposition temperature	45 °C
Chromatographic conditions	
Temperature injector	200 °C
Temperature detector	300 °C
Column	HP-5; 30 m × 0.53 mm ID × 1.0 µm
Column flow	(He) 1.0 ml/min
Split	1:20
Oven temperature program	40 °C, 3 min; 5 °C/min up to 110 °C; 20 °C/min up to 270 °C

LITERATURE:

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