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Chromatographic on-line detection of bioactives in food

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ABSTRACT:

Findings were focused on the anti-oxidative activity of numerous fruits and vegetables by means of an on-line HPLC radical scavenging detection method. The reactant used was the $ABTS^{\bullet+}$ green radical cation. The system has been optimized in terms of reactor design, and chemical reactions kinetics. It has been qualified to classify molecules in order of their increasing activity to scavenge exogenous radicals. It may be used as a powerful high resolution screening tool to investigate the radical scavenging activities of natural plants. Bioassays consisting in cellular *in vitro* antioxidant assay using pancreatic β-cells have been used to confirm the bioactivity of the selected micronutrients. This study demonstrated that it is possible to screen at the molecular level, the bioactivity of numerous natural samples and to point out the richness of the local biodiversity in terms of natural resource of functional food ingredients usable for their potential benefits for consumer's health, wellbeing and well-aging.

Key words: HPLC radical scavenging detection method, bioactivity of natural samples,

BACKGROUND:

The micronutrients present in functional foods are now widely recognized as being a powerful tool for the modulation of many diseases, such as diabetes, cancer or neurodegenerative diseases, and to reduce the risk of severe complication of them. When the disease is based on an inflammatory process, a diet containing anti-inflammatory compounds may be highly advisable for the consumer [1; 2; 3; 4]. The classical method for bioactive natural compound discovery relies on a time-consuming bio-guided fractionation procedure. Inflammation processes are mainly originating from oxidative stress and radical generation

within the cell. Major bioassays commonly used to detect an anti-inflammatory activity of a newly discovered compound are based on chemical methods which do not reflect the capacity of the compound to enter into the cell and act within the metabolic pathway to avoid chemically reactive oxygen species (ROS) to react on the bio-pathways.

The object of this paper is to present a method able to avoid the costly and long-timing bio-guided fractionation and to confirm the detected activity of the active fractions by cell-based bioassays. As radical scavengers are able to modulate the inflammatory process, the chosen cell targets were pancreatic β-cells producing insulin and responsible of many diabetes syndromes.

MATERIALS AND METHODS:

Material: The chromatographic system was a Dionex HPLC System consisting in a Dionex Ultimate Autosampler WPS3000FC, a Dual Gradient Pump 3600 SD, a Diode Array Detector 3000(RS), a Variable Wavelength Detector 3100. The chromatographic column was an ACE C18-PFP (250 x 4.,6 mm; 5 µm), the flow rate was of 1 mL/min and the injection volume was of 20 µL. The mobile phase was a gradient of a solvent (A) consisting in Milli-Q a mixture of water and Trifluoroacetic acid (99.9/0.1, v/v) and a solvent (B) consisting in Acetonitrile. The gradient was as indicated in Figure 1.

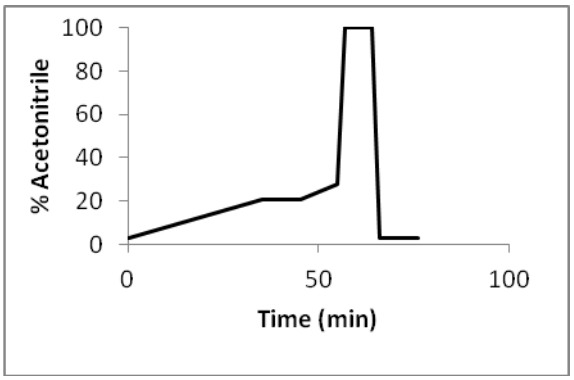

Figure 1. Concentration of acetonitrile during the HPLC gradient of elution

After their separation, the eluted compounds were diverted to a DAD detector (200 nm <λ<800 nm) and to a reactor in which a second pump injected, at a flow rate of 0.5 mL/min, a solution of $ABTS^{\bullet+}$ radical cation. The mixture was guided through a 25 meter long reaction coil with 0.25 mm internal diameter to a second UV detector, where decolourization is monitored at 412 nm.

Reagents: 2.5mM Potassium persulfate and 7 mM ABTS were dissolved in milli-Q water, generating the radical cation $ABTS^{\bullet+}$ overnight. This solution was used within 4 days, diluted in phosphate buffer saline buffer (PBS) (8 mM pH 7.4). A Trolox calibration curve between 10 and 250 µM was obtained (R^2 > 0.98) in order to quantify the anti-oxidant capacity of the separated compounds, which are expressed in µmole of Trolox equivalent per 100 g of fresh matter.

Food product: the food products were given by the Interprofession des Fruits et Légumes (68127 Sainte Croix en Plaine, France). It consists in red cabbage, onion, quince, cherries (varieties *Regina* and *Folfer*), spinach, black raddish, strawberries (varieties *Florence* and *Salsa*), celeriac, tomato, carrots, asparagus and zucchini. They were freeze

dried, defatted with hexane, submitted to a CH_2Cl_2 extraction to remove the non-polar fraction and extracted with methanol to recover the polar fraction which was subsequently submitted to a SPE purification dedicated to remove the sugar content. The obtained extracts were named Sugar Free Polar Extracts (SFPE).

The bioassays based on a modified Cellular Antioxidant Activity assay [5], were performed with pancreatic β-cell (RINm5f) chosen for their high sensitivity to oxidative stress [6]. The cells were seeded at 100000 cells/well, and were submitted first (24 h) to di-acetylated 2′,7′-dichlorofluorescein (DCFH-DA, 60 µM) and to different concentrations of the SFPE extracts, dissolved in RPMI-1640 medium.After rinsing the cells with HBSS buffer, they were exposed to the oxidative stress with Umulin NPH (25 UI/mL). Indeed, insulin exposure has been shown to induce ROS production, which is mainly represented by H_2O_2 [7], or anion superoxyde. From the initiation of the oxidative stress, the fluorescence signal (λ_{Ex} = 485 nm; λ_{Em} = 538 nm) was recorded during 100 min. The CAA value was calculated as follows:

$$CAA = 100 \cdot \left[1 - \frac{\int Sample - \int Blank}{\int Control - \int Blank} \right]$$

RESULTS:

The whole SFPE extracts obtained by the proposed method of extraction and purification were evaluated thanks to the TEAC and ORAC methods of analysis. These two methods are already well documented in the relevant literature [8; 9]. They allow highlighting different processes occurring during the radical scavenging pathway. TEAC focuses on the ability of compounds to transfer protons towards the $ABTS^{\bullet+}$ radical cation initiated first through electron transfer, whereas ORAC is more devoted to the detection of hydrogen atom transfer towards peroxyl radicals [10; 11]. TEAC signals is measured as a decrease of an optical absorption, whereas ORAC one is detected as a reduction of the fluorescent signals. Although these two methods are very different, TEAC being an end-point measure and ORAC taking kinetics into account, they gave the same indication concerning the radical-scavenging capacities of the SFPE analyzed.

Figure 2 shows clearly (higher the signal, higher the activity) that the most active SFPE extract is the one obtained from red cabbage. One can classify the extracts within three categories, the most active one, above the green line, at 300 µmole/100 g of fresh food (Cherry (var. *Regina*), quince, red cabbage and onion), the less active, below the red line, less than 10 µmole/100 g of fresh food (asparagus, zucchini and carrot) and the medium active, between 300 and 10 µmole/100 g of fresh food (Cherry (var. *Folfer*), strawberry (var. *Salsa* and *Florence*), tomato, celeriac, spinach and black radish). Whatever the chemical method used (TEAC or ORAC) the classification remains the same. In the same way, the intensities of the recorded signals were in the same order of magnitude, in between 0 and 1500 µmol of trolox equivalent/100g of fresh matter. The last observation was the effect of the variety of the analyzed food. Within the two strawberry varieties, it appears clearly, whatever the method used, that the *Florence* was at least twofold more active than the *Salsa* one. In the same way, the *Regina* variety of cherry was also two times more active than the *Folfer* one. One should certainly also observe an equivalent effect when changing the agricultural and/or the meteorological conditions of growth. Particular attention should be given not to

generalize these results to any other food of the same type grown in other ways and habits in other regions than Alsace.

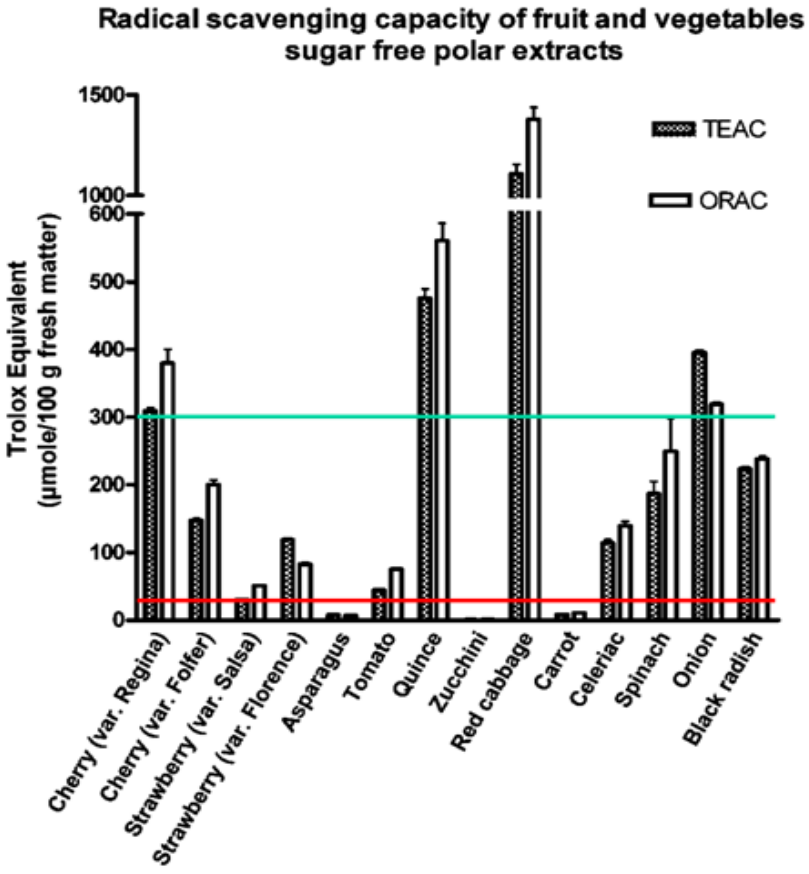

Figure 2. Chemical (TEAC and ORAC) radical scavenging activities of the different SPE extracts obtained with different regional food from the region Alsace. The highest activities are above the green line, the lowest activities are below the red line.

The bioassay realized with the same extracts allowed calculating an IC_{50} which has to be considered as being the concentrations of SFPE extracts able to reduce about 50% the fluorescence emitted by the de-acytelated and oxidized DCFH compound used as a fluorescent probe. IC_{50} obtained from the different foods presented the same classification as the chemical tests (TEAC and ORAC).

Figure 3 shows the classification as obtained with the bioassays (lower the IC_{50}, higher the activity), indicating that the better activity, below the green line, with IC_{50} lower than 35 mg of fresh matter/mL, was presented by the SFPE extracts from Cherry (var. *Regina*), quince, red cabbage and onion, the lower activity being, above the red line with IC_{50} higher than 160 mg of fresh matter/mL presented by asparagus and zucchini and finally the medium activities being presented by SFPE of carrot, cherry (var. *Folfer*), strawberry(var. *Salsa* and *Florence*), tomato, celeriac, spinach and black radish having IC_{50} between 35 and 160 mg of fresh matter/mL. Except carrots, all the studied foods presented the same classification when tested with the two different chemical methods (TEAC and ORAC) and by the cellular bioassay. This may suggest that the carrot extract does not exert its bioactivity through a radical scavenging based mechanism. It is quite rare that such accordance between chemistry and biology is presented.

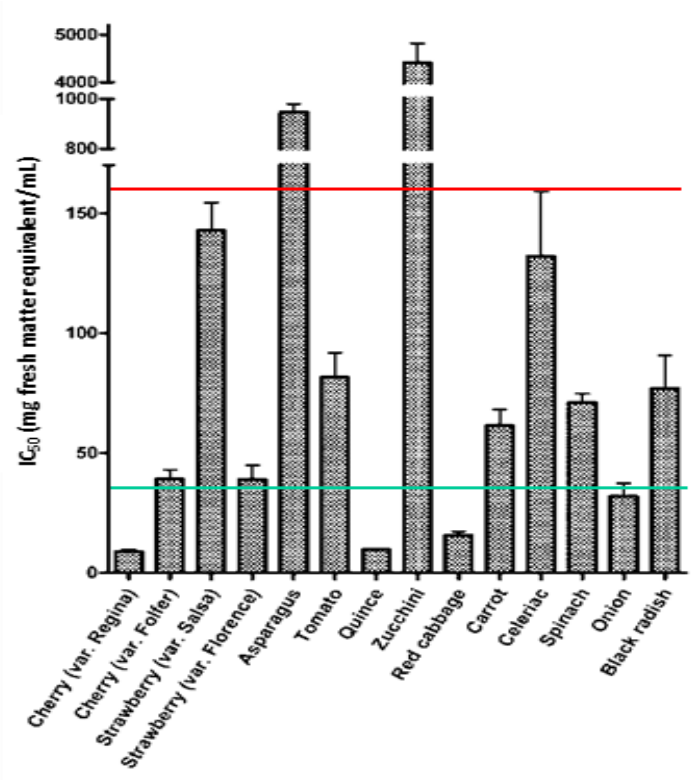

Figure 3. Biological radical scavenging activities of the different SFPE extracts obtained with different regional food from the region Alsace. The highest activities are below the green line, the lowest activities are above the red line.

This work allows the determination of the most active SFPE extract, but it gives no information about the chemical structure of the active species present in the plant tested. To get more information one should perform expensive and time-consuming bio-guided fractionation, again and again. By cons, the use of a chromatographic separation hyphenated with a post-column reactor mixing the eluted mobile phase with the $ABTS^{\bullet+}$ radical cation, can allow a continuous monitoring of the radical-scavenging activity of compounds eluted from the column (method adapted from Koleva et al, 2000 [12]).

Figure 4 presents the 2-D chromatogram of a mixture of several polyphenols at the same concentration. One can observe that the chromatogram on the top, , gives indication about eluted compounds which absorb at 280 nm. The chromatogram situated at the lower part of the figure indicates the decolourization of the $ABTS^{\bullet+}$ radical cation, which degrades from its green precursor color to a colorless form which does not absorb this wavelength.

A negative peak is a proof that a compound having radical scavenging activity elutes out of the chromatographic column. The area of the chromatographic negative peak is proportional to the radical-scavenging activity of the considered compound, which means a quantitative appreciation of each compounds activity can be made. One can now classify these chemical standards as a function of their activity as being the slope of the regression lines calculated when analyzing several concentration of these compounds. Among the tested chemical standards, the less active was ferulic acid, gallic acid being the most active. Further experiments proved that epigallocatechin gallate was much more active that gallic acid. Analysis of SFPE extracts from regional fruits and vegetables are presented in figure 5 (apple) to 7 (cherry). The presented chromatograms allow the quick and easy detection of all the radical-scavenging compounds eluting out of the column. On figure 5, two negative peaks

(tr = 32 min and 34 min) are the proof of two new bioactives in Weirouge apple. These two bioactives are yet unknown just because they do not have any corresponding positive peak due to the lack of chromophore that would allow their UV detection. The negative peaks are easily collectable by an automatic 96 wells micro-plate collector. The isolated compounds being of high purity could be identified thanks to high resolution spectrometries such as 500 MHz ^{1}H and ^{13}C NMR, HRMS and IRFT. The identities of the detected negative peaks are indicated on the positive chromatograms of quince (fig 6) and cherry (fig 7).

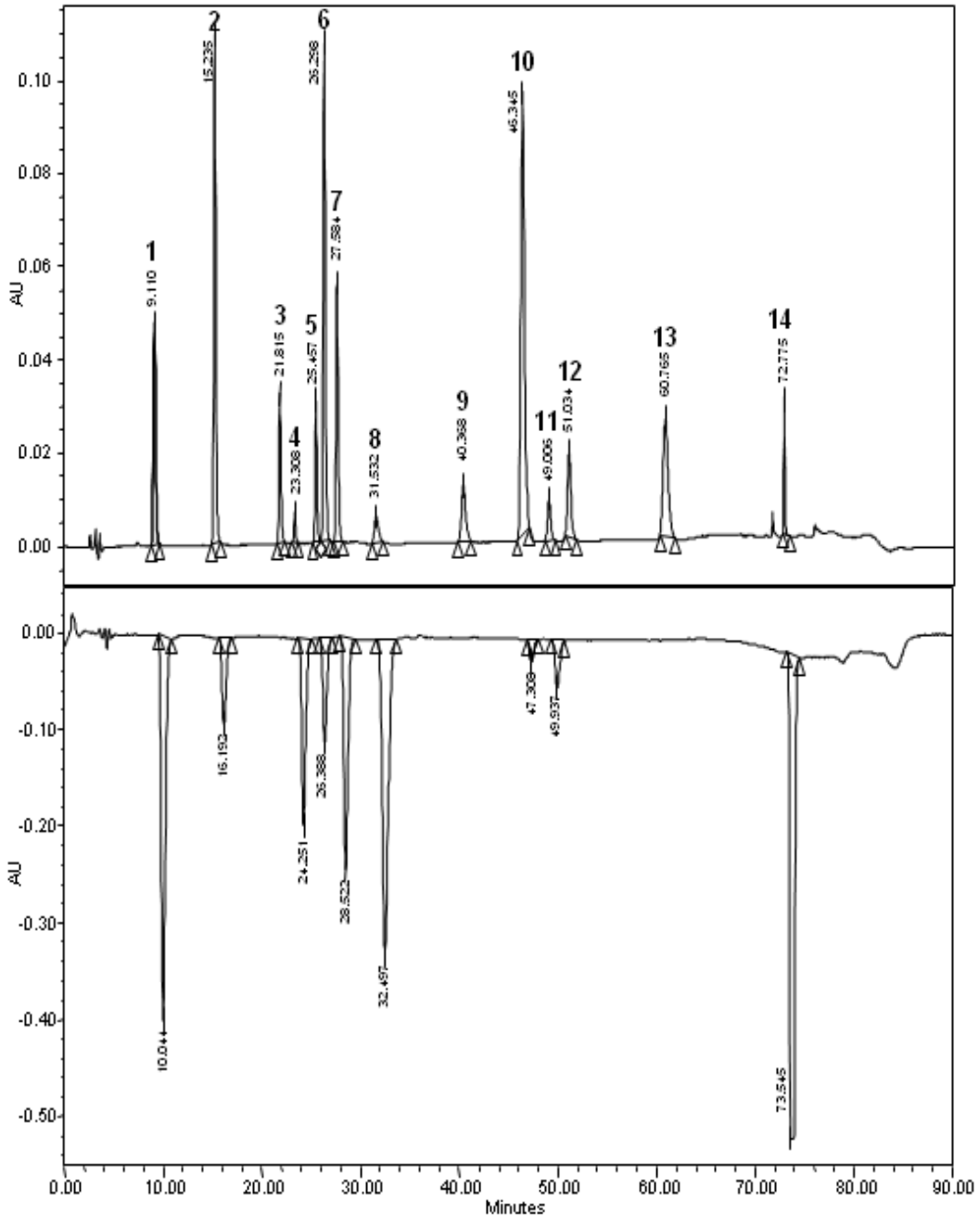

Figure 4. Chromatographic separation of a mixuture of several polyphenols, each of the same concentration. 1:gallic acid, 2:protocatechuic acid, 3:p-hydroxybenzoic acid, 4:catechin, 5:chlorogenic acid, 6:vanillic acid, 7:caffeic acid, 8:epicatechin, 9:p-coumaric acid, 10:ferulic acid, 11:sinapic acid, 12:m-coumaric acid, 13:o-coumaric acid and 14:trolox.

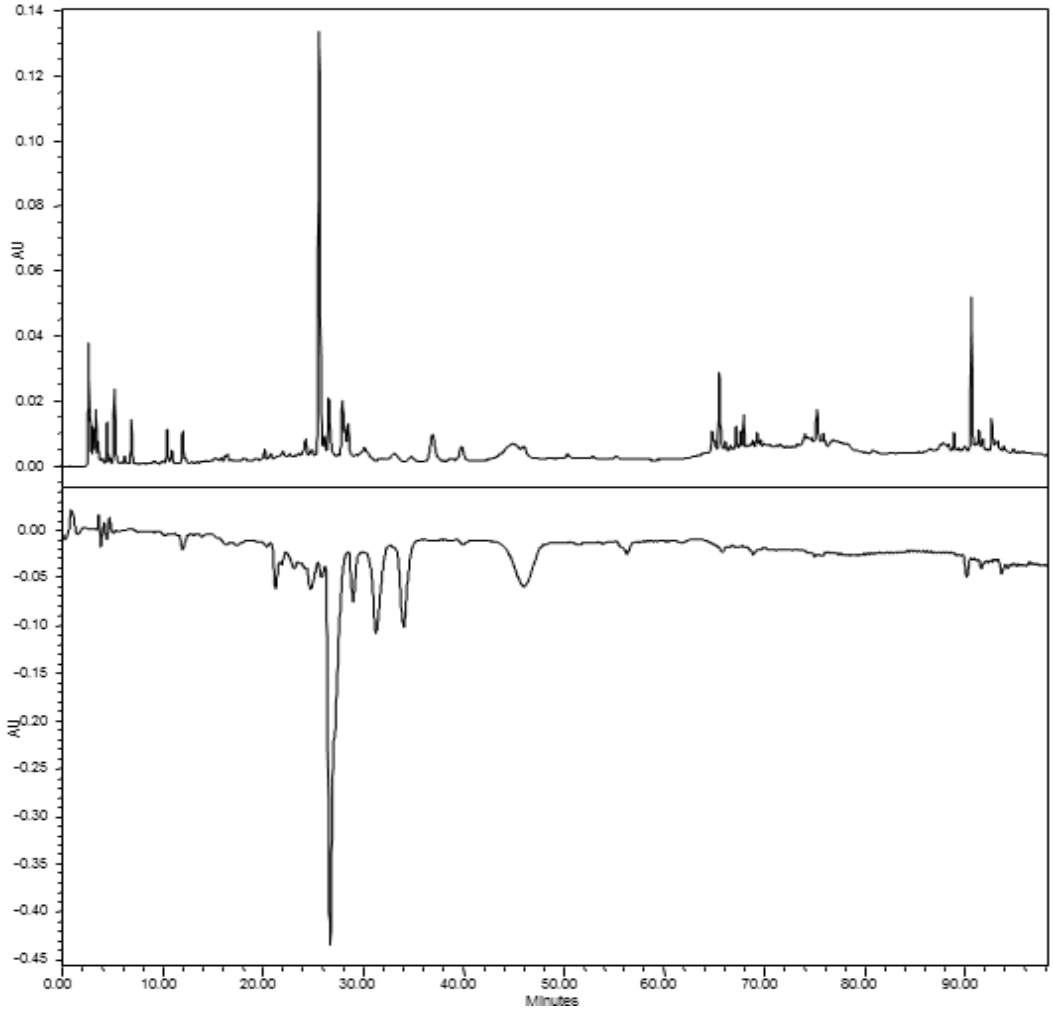

Figure 5. 2-D chromatogram of the SFPE extract from a regional apple (var. *Weirouge*).

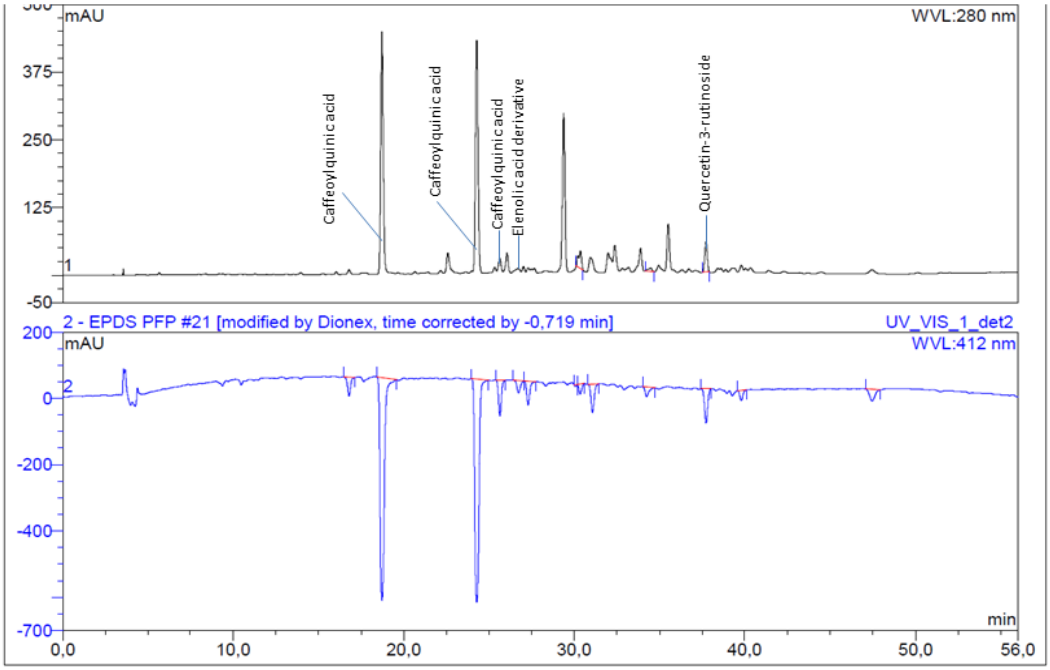

Figure 6. 2-D chromatogram of the SFPE extract from a regional quince (var. *Common*).

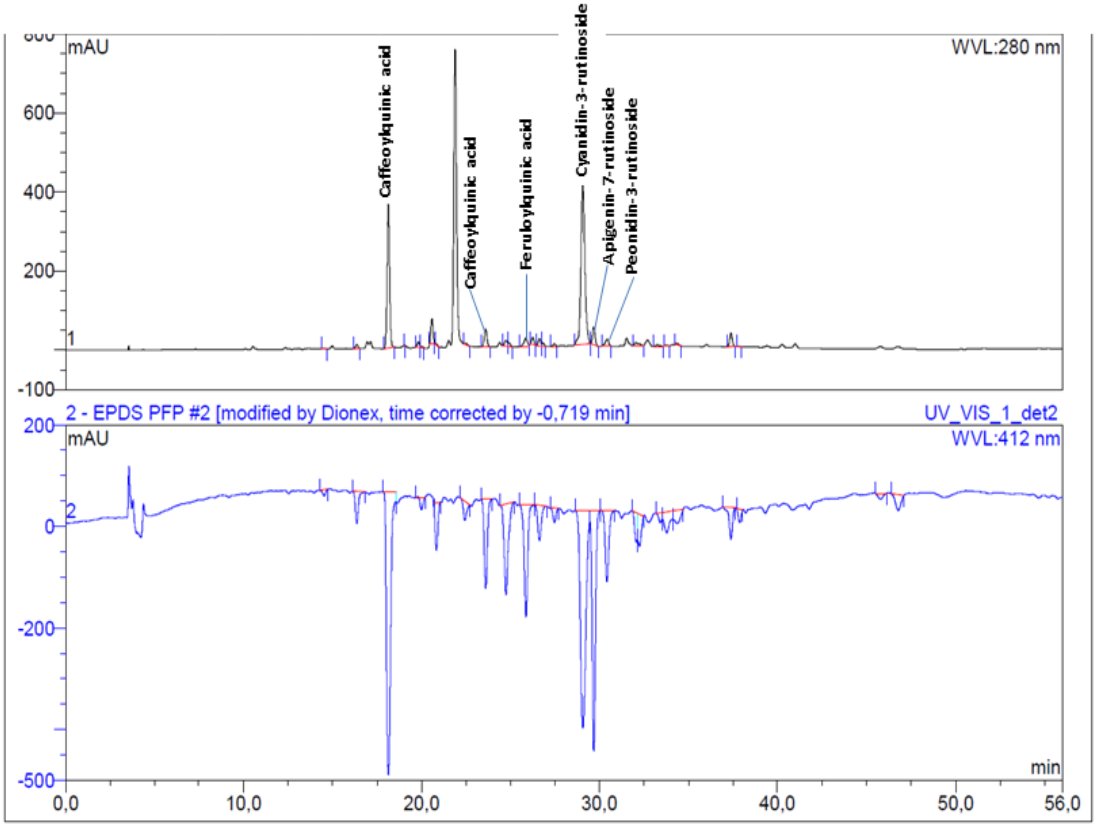

Figure 7. 2-D chromatogram of the SFPE extract from a regional cherry (var. *Regina*).

CONCLUSION:

Thanks to this method, it is now possible to quickly detect new natural bioactive compounds. It is a high resolution screening method that allows a physical isolation of bioactive molecules and to determine their exact chemical structures. That is of great interest when one wishes to make supplemented foods in order to create new functional foods.

Abbreviations: ABTS•+: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), CAA: Cellular Antioxidant Activity, DAD: Diode Array Detector, DCFH-DA: di-acetylated 2′,7′-dichlorofluorescein, HBSS: Hank's Balanced Salt Solution, HPLC: High performance liquid chromatography, NPH: Neutral protamine Hagdorn, ORAC: Oxygen radical absorbance capacity, ROS: Reactive oxygen species, RPMI: Roswell Park Memorial Institute, SFPE: Sugar free polar extract, TEAC: Trolox equivalent antioxidant capacity

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