Universitat de Lleida

Development and application of analytical methods to characterise processed fruit and vegetable products

Antoni del Pino Rius

http://hdl.handle.net/10803/459296

ADVERTIMENT. L'accés als continguts d'aquesta tesi doctoral i la seva utilització ha de respectar els drets de la persona autora. Pot ser utilitzada per a consulta o estudi personal, així com en activitats o materials d'investigació i docència en els termes establerts a l'art. 32 del Text Refós de la Llei de Propietat Intel·lectual (RDL 1/1996). Per altres utilitzacions es requereix l'autorització prèvia i expressa de la persona autora. En qualsevol cas, en la utilització dels seus continguts caldrà indicar de forma clara el nom i cognoms de la persona autora i el títol de la tesi doctoral. No s'autoritza la seva reproducció o altres formes d'explotació efectuades amb finalitats de lucre ni la seva comunicació pública des d'un lloc aliè al servei TDX. Tampoc s'autoritza la presentació del seu contingut en una finestra o marc aliè a TDX (framing). Aquesta reserva de drets afecta tant als continguts de la tesi com als seus resums i índexs.

ADVERTENCIA. El acceso a los contenidos de esta tesis doctoral y su utilización debe respetar los derechos de la persona autora. Puede ser utilizada para consulta o estudio personal, así como en actividades o materiales de investigación y docencia en los términos establecidos en el art. 32 del Texto Refundido de la Ley de Propiedad Intelectual (RDL 1/1996). Para otros usos se requiere la autorización previa y expresa de la persona autora. En cualquier caso, en la utilización de sus contenidos se deberá indicar de forma clara el nombre y apellidos de la persona autora y el título de la tesis doctoral. No se autoriza su reproducción u otras formas de explotación efectuadas con fines lucrativos ni su comunicación pública desde un sitio ajeno al servicio TDR. Tampoco se autoriza la presentación de su contenido en una ventana o marco ajeno a TDR (framing). Esta reserva de derechos afecta tanto al contenido de la tesis como a sus resúmenes e índices.

WARNING. Access to the contents of this doctoral thesis and its use must respect the rights of the author. It can be used for reference or private study, as well as research and learning activities or materials in the terms established by the 32nd article of the Spanish Consolidated Copyright Act (RDL 1/1996). Express and previous authorization of the author is required for any other uses. In any case, when using its content, full name of the author and title of the thesis must be clearly indicated. Reproduction or other forms of for profit use or public communication from outside TDX service is not allowed. Presentation of its content in a window or frame external to TDX (framing) is not authorized either. These rights affect both the content of the thesis and its abstracts and indexes.

UNIVERSITAT DE LLEIDA

ESCOLA TÈCNICA SUPERIOR D'ENGINYERIA AGRÀRIA

DEPARTAMENT DE QUÍMICA

DEVELOPMENT AND APPLICATION OF ANALYTICAL METHODS TO CHARACTERISE PROCESSED FRUIT AND VEGETABLE PRODUCTS

Antoni del Pino Rius

Supervised by

Dr. Mercè Balcells Fluvià

Dr. Jordi Eras i Joli

Lleida

2017

Agraïments

M'agradaria dedicar la finalització d'aquesta Tesis Doctoral a totes aquelles persones que m'han acompanyat i donat suport, consell i ànim al llarg d'aquest procés, sense les quals hauria estat impossible assolir aquest l'objectiu.

A els meus directors de tesis el Dr. Jordi Eras i la Dra. Mercè Balcells, per oferir-me els seus valuosos coneixements i experiència professional, així com ajudar-me i encoratjar-me en tot moment d'aquesta investigació. De la mateixa manera al Dr. Ramon Canela i la Dra. Gemma Villorbina per la seva col·laboració i suport al llarg d'aquests anys.

A tota aquella gent que ha estat al meu costat en el dia a dia al laboratori al llarg d'aquests anys. De veritat, moltes gràcies a tots pels moments viscuts tant a dins com a fora del laboratori, per ajudar-me quan ho he necessitat i per fer que venir cada dia al laboratori fos un lloc on riure i compartir bons moments.

A els membres que formen part del departament de química i dels serveis cientificotècnics de la Universitat de Lleida, pels coneixements i experiència transmesos durant aquests anys.

I would like to thank Dr Mondello to allow me to work in your research group. I spent there a very wonderful period of my thesis and I have happy memory of all the members of your group, specially of Dr Cacciola, Dr Giuffrida and Dr Utczás with I am very grateful for.

A la gent del COS per acompanyar-me i recolzar-me durant el tram final d'aquesta Tesis. Així com a la gent de Zucasa pels mesos compartits i l'amistat brindada.

A tot el amics que m'han recolzat i compartit els moments més o menys durs durant aquests anys, una part de la Tesis es vostra.

A el Pol simplement saps que gran part d'aquesta Tesis es teva. Sense tu no hauria estat possible assolir aquest objectiu. Gràcies, per la teva amistat i ajut durant tots aquests anys.

A els meus pares pel seu amor i suport incondicional i continu. Ells són sense dubte els artífex d'aquesta Tesis, per aquest fet m'agradaria dedicar-vos-la.

Summary.

This thesis presents an approach to the development and application of analytical methodologies to characterise metabolite compounds in processed fruit and vegetable products from the agroindustry.

In this line, in this thesis were developed ultra-performance liquid chromatography coupled to photodiode array and tandem mass spectrometry detectors(UPLC-PDA-MS/MS) methods to determine carotenoids and chlorophylls and their derivatives in processed fruit and vegetable products. The method developed to analyse carotenoids was applied to study the changes in the carotenoid profile of commercial monovarietal fruit juices. The developed method allowed us to identify up to 27 carotenoids in apple, peach and pear products. Moreover, the methodology allows us to differentiate the carotenoid profiles from commercial juices and homemade fresh peach and pear juices, as well as to study the rearrangements of 5,6- to 5,8-epoxycarotenoids. The method developed to analyse chlorophyll and their derivatives was applied to assess the fate of chlorophylls in processed vegetable products. The method allowed to evaluate the degree of processing in teas of different origin and quality. In processed green vegetable and fruit products were determined mainly pheophytins, pheophorbides and pyro derivatives and in several products were also detected copper-chlorophyll derivatives used as food coloring agents.

Also various methodologies and techniques were applied to characterise processed fibres obtained from juice industry by-products. The phenolic compounds, including bound phenolic compounds, of the fibres were determined and a comparison of its content and profile in lyophilised fresh fruit was established. Additionally metabolite profile and content of the fibres was characterised, and compared with data reported in previous literature for the corresponding fresh fruit. In this study primary metabolites such as sugars, sugar alcohols, amino acids, organic acids and fatty acids and secondary metabolites such as phytosterols, triterpenes, fatty alcohols, alkanes and carotenoids were analysed.

Finally, with the aim of obtain a methodology to authenticate monovarietal fruit juices, were used nuclear magnetic resonance spectroscopy (NMR) to analyse primary metabolites and UPLC-PDA-MS/MS to determine phenolic compounds combined chemometric tools such as principal component analysis. The PCA from combined data of primary metabolites and phenolic compounds allowed a satisfactory discrimination of Alejandrina, Conference, and Blanquilla cultivars in pear juices and between Miraflores and Spring Lady cultivars in peach purees.

Resum.

En aquesta tesi es presenta el desenvolupament i aplicació de metodologies analítiques per caracteritzar metabòlits en productes de fruita i verdura processats.

En aquesta línia es van desenvolupar metodologies de cromatografia líquida d'ultra-rendiment acoblada a un detector espectrometria de masses en tàndem i un de matriu de fotodíodes (UPLC-PDA-MS/MS) per determinar els carotenoides i clorofil·les i els seus derivats en productes processats de fruites i verdures. La metodologia per analitzar els carotenoides es va aplicar per estudiar en els sucs de fruites monovarietals els canvis en el perfil de carotenoides. El mètode ens va permetre identificar fins a 27 carotenoides en els diferents productes de poma, préssec i pera. D'altra banda, la metodologia permet diferenciar els perfils de carotenoides entre sucs comercials i frescos de préssec i pera, així com estudiar els reordenaments de 5,6- a 5,8-epoxicarotenoides. La metodología per analitzar les clorofil·les i els seus derivats es va aplicar per avaluar la transformació de les clorofil·les en productes vegetals processats. El mètode permet avaluar el grau de processament en tes de diferent origen i qualitat. En els productes vegetals processats es van determinar principalment feofitines, feofórbidos i els seus piro-derivats, també es van detectar derivats de la clorofil·la tractada amb coure utilitzats colorants alimentaris.

També varies metodologies i tècniques es van utilitzar per caracteritzar fibres processades obtingudes a partir de subproductes de la indústria del suc. Es van determinar els compostos fenòlics de les fibres i es va establir una comparació amb el contingut i perfil trobat en la fruita fresca liofilitzada. A més a es va caracteritzar el perfil i el contingut de metabòlits de les fibres i es va comparar amb les dades reportades en la literatura per a fruita fresca. En aquest estudi es van analitzar els metabòlits primaris com ara sucres, alcohols de sucre, aminoàcids, àcids orgànics i àcids grassos i metabòlits secundaris com ara fitosterols, triterpens, alcohols grassos, alcans i carotenoides.

Finalment, amb l'objectiu d'obtenir una metodologia per autentificar els sucs monovarietals de fruites, es van utilitzar la ressonància magnètica nuclear (metabòlits primaris) i la *UPLC-PDA-MS/MS* (compostos fenòlics) en la detecci combinats amb eines quimiomètriques com ara l'anàlisi de components principals (*PCA*). El *PCA* a partir de dades combinats va permetre una discriminació satisfactòria de les varietats Alexandrina, Conference i Blanquilla en sucs de pera i entre les varietats Miraflores i Spring Lady en purés de préssec.

Resumen.

En esta tesis se presenta el desarrollo y aplicación de metodologías analíticas para caracterizar metabolitos en productos en productos de fruta y verdura procesados.

En esta línea se desarrollaron metodologías de cromatografía líquida de ultra-rendimiento acoplada a un detector espectrometría de masas en tándem y uno de matriz de fotodiodos *(UPLC-PDA-MS/MS*) para determinar los carotenoides y clorofilas y sus derivados en productos procesados de frutas y verduras. El método analítico desarrollado para analizar los carotenoides se aplicó para estudiar en los zumos de frutas monovarietales los cambios en el perfil de carotenoides. El método desarrollado nos permitió identificar hasta 27 carotenoides en los diferentes productos de manzana, melocotón y pera. Por otra parte, la metodología permite diferenciar los perfiles de carotenoides entre zumos comerciales y frescos de melocotón y pera, así como estudiar los reordenamientos de 5,6- a 5,8-epoxicarotenoides. El método analítico desarrollado para analizar las clorofilas y sus derivados se aplicó para evaluar la transformación de las clorofilas en productos vegetales procesados. El método permite evaluar el grado de procesamiento en tés de diferente origen y calidad. En los productos vegetales procesados se determinaron principalmente feofitinas, feofórbidos y sus piro-derivados, también se detectaron derivados de la clorofila tratada con cobre utilizados como colorantes alimenticios.

También, diversas metodologías y técnicas se utilizaron para caracterizar fibras procesadas obtenidas a partir de subproductos de la industria del zumo. Se determinaron los compuestos fenólicos de las fibras y se estableció una comparación con el contenido y perfil encontrado en la fruta fresca liofilizada. Adicionalmente se caracterizó el perfil y contenido de metabolitos de las fibras y se comparó con los datos reportados en la literatura para fruta fresca. En este estudio se analizaron los metabolitos primarios tales como azúcares, alcoholes de azúcar, aminoácidos, ácidos orgánicos y ácidos grasos y metabolitos secundarios tales como fitoesteroles, triterpenos, alcoholes grasos, alcanos y carotenoides.

Por último, con el objetivo de obtener una metodología para autentificar los zumos monovarietales de frutas, se utilizaron la resonancia magnética nuclear (metabolitos primarios) y la *UPLC-PDA-MS/MS* (compuestos fenólicos) combinados con herramientas quimiomètricas tales como el análisis de componentes principales (*PCA*). El *PCA* a partir de datos combinados permitió una discriminación satisfactoria de los cultivares Alejandrina, Conference y Blanquilla en zumos de pera y entre los cultivares Miraflores y Spring Lady en purés de melocotón.

INDEX

CHAPTER 1. INTRODUCTION

1. Introduction.

Fruit intake is highly-recommended. Approximately one third of total daily food intake should include fruits and vegetables distributed in each meal and also as snacks [1]. But fresh fruits and vegetables are highly perishable products due to their high water content (over 90 %) and once harvested they begin to undergo a rapid process of degradation, loss of consumer appeal and an increase of its potential microbial spoilage [2]. In this context, processing technologies are being utilised to transform these perishable fruits and vegetables into safe, delicious and stable products. In addition, processing technologies allow the consumption of fruit and vegetable products during all the year independently of the growing area of the fruit or vegetable.

Fruit and vegetables are attractive foods due to their flavour, appearance and they have nutritious components that are important contributors to a healthy diet. In these quality parameters a diverse variety of organic compounds are involved which are the product of primary and secondary metabolism [3]. Some non-volatile primary metabolites are essential components of taste [4], which combined with smell provided by volatile compounds, is associated to flavour perception [5]. Also, some primary and secondary metabolites play an important role in the colour of fruit and vegetables, which directly influences their appearance and attractiveness. In recent years, the role of some secondary metabolites as protective dietary constituents has become an increasingly important area of human nutrition research. Unlike the traditional vitamins they are not essential for short-term well-being, but there is increasing evidence that modest long-term intakes can have favourable incidence against cancers and many chronic diseases [1, 6]. The amount of these metabolites improves and increases as fruits approach to their maturity peak and decreases after their harvesting. In these circumstances, processing converts fruits and vegetables in processed products with a stable colour, flavour and nutrients, reduces the loss of nutritious components and moisture and controls the growth of microorganisms. However, depending on the processing technology used, the primary and secondary metabolites are maintained in a different extend.

This introductory chapter first contains the basic concepts and description of the main processed fruit and vegetables and secondly a description of non-volatile primary and secondary metabolites. The second part delves into the effect of processing over each type of compound, as well as the analytical methodologies established to analyse them in fruit and vegetable products. The experimental section of this thesis consists of three chapters, the first is *Development of UPLC-PDA-MS/MS methods to determine the carotenoids and chlorophylls and their derivatives in processed fruit and vegetable products,* the second is *Characterisation of primary and secondary metabolites in processed fibres from juice industry* and the last is *Analysis of primary metabolites and polyphenolic compounds to authenticate monovarietal fruit juices.*

1.1. Processed fruit and vegetables

In some countries agriculture is the mainstay of the economy so that it should be no surprise that agricultural industries and related activities can account for a considerable percentage of their output. Fruit and vegetable processing activities have been set up, or are established in countries for one or another of the following reasons [7]: diversification of the economy, reduction of imports, stimulation of agricultural production, generation of both, rural and urban employment, reducing of fruit and vegetable losses and generation of new income sources for farmers.

The growth in global per capita income has spurred growth in global demand for food. In addition, the rising global health trend has increased consumer demand for high-quality and more diverse fruit and vegetable products [8]. Simultaneously, improvements in logistics and production processes have enabled the industry's larger multinational companies to increase their production and more efficiently distribute their products over geographically disperse markets. Under these circumstances, and considering that fruit and vegetable juice industries are very important in Europe where the processing industry of fruits and vegetables has great historical roots [9], it is understandable that fruit juice exports in 2013 were led by Developed Europe (Figure 1). Other areas that stand out in terms of export are Latin America and Caribbean in fruit and vegetable juices and Eastern Asia in prepared or preserved vegetables. In terms of imports the richer developed zones as Europe and North America dominate. However, processed fruit and vegetable products demand has grown strongly in the developing world over the past ten years due to industrialisation, urbanisation of the population and increased incomes [10]. This has led to an increase in demand for processed fruit and vegetable products as the developing world's population has increased its consumption ratio of processed to unprocessed food [8].

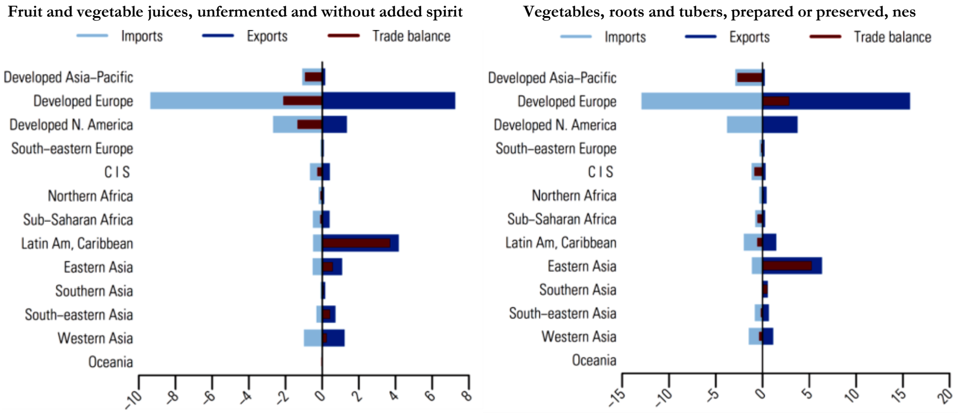


Figure 1. Trade balance of fruit and vegetable juices, unfermented and without added spirit by MDG (Millennium Development Goals) regions in 2013 [10].

The main objective of fruit and vegetable processing is to supply wholesome, safe, nutritious and acceptable food to consumers throughout the year. Whilst most fruit and vegetables should be eaten fresh, processed fruit and vegetables can be acceptable alternatives. They come in many forms, including [11]:

- Fruit and vegetable juices and certain similar products.
- Canned fruit pulp and vegetables.
- Preserves, jams and jellies.
- Dried fruit and vegetables.
- Fruit and vegetable fibres.

1.1.1. Fruit and vegetable juices and certain similar products.

Within the European union (EU), fruit juice is regulated by Council Directive 2001/112/EC [12] relating to fruit juices and certain similar products intended for human consumption, last amended by Directive 2012/12/EU of the European Parliament 19 April 2012 [13]. Vegetable juices, are not covered by the fruit juice directive, however, due to the many similarities, for best practice it would seem sensible to treat them in the same way [14]. In this document the characteristics and names of the regulated products are defined. They include fruit juice (also referred as juice not from concentrate), fruit juice from concentrate, concentrated fruit juice, water extracted fruit juice, dehydrated/powdered fruit juice and fruit nectar. Fruit nectar is obtained by adding water with or without the addition of sugars and /or honey to the products above mentioned, to fruit puree and/or to fruit puree and/or to a mixture of those products [13]. Since various manufacturers of industrial transformation of fruit have collaborated in this thesis providing extensive range of regulated products and other products (referred in Council Directive 2001/112/EC as raw materials) such as juices, concentrates and purées, the main products and industrial processes for their production are described below in more detail [15, 16]. These detailed products are puree, juice not from concentrate (*clear or cloudy*) and concentrated juice (*clear or cloudy*).

Juice processing involves various stages some of which are common to all products whilst some others are specific to each product. Figure 2 shows a general outline of the steps involved to obtain fruit juices and purees. Next, the main steps involved in obtaining each product are disclosed in more detail. Although there are differences between each processing plant, processing usually begins with the washing and sorting of the fruit to ensure it is cleaned and graded for use. Washing is typically conducted with a high-pressure soak or spray system [17]. Sometimes it involves a surfactant or detergent, which is added in order to release stubborn attached soil from the fruit. In some fruits, processing requires a high-quality wash to ensure the safe removal of microorganisms responsible for mycotoxin formation. For example, in apple it is necessary to avoid the formation of patulin [18]. To obtain their juice, fruits need to be milled; crushed or chopped. Other common stages for certain types of products, are bleaching in the case of some vegetables and destoning for stone fruits [19].

Puree.

According to the Council Directive 2001/112/EC fruit puree is defined as a raw material and described as the fermentable but unfermented product obtained by sieving the edible part of whole or peeled fruit without removing the juice [12]. Puree retains all of the juice and a large proportion of the fibrous matter naturally found in the fruit. Only seeds or stones and, sometimes, excess insoluble fibres are removed during the processing. Once this has been done, the fruit is placed in an extractor to remove any unwanted fibre, skin particles or remaining seeds, leaving the smooth pulp and juice of the fruit [20].

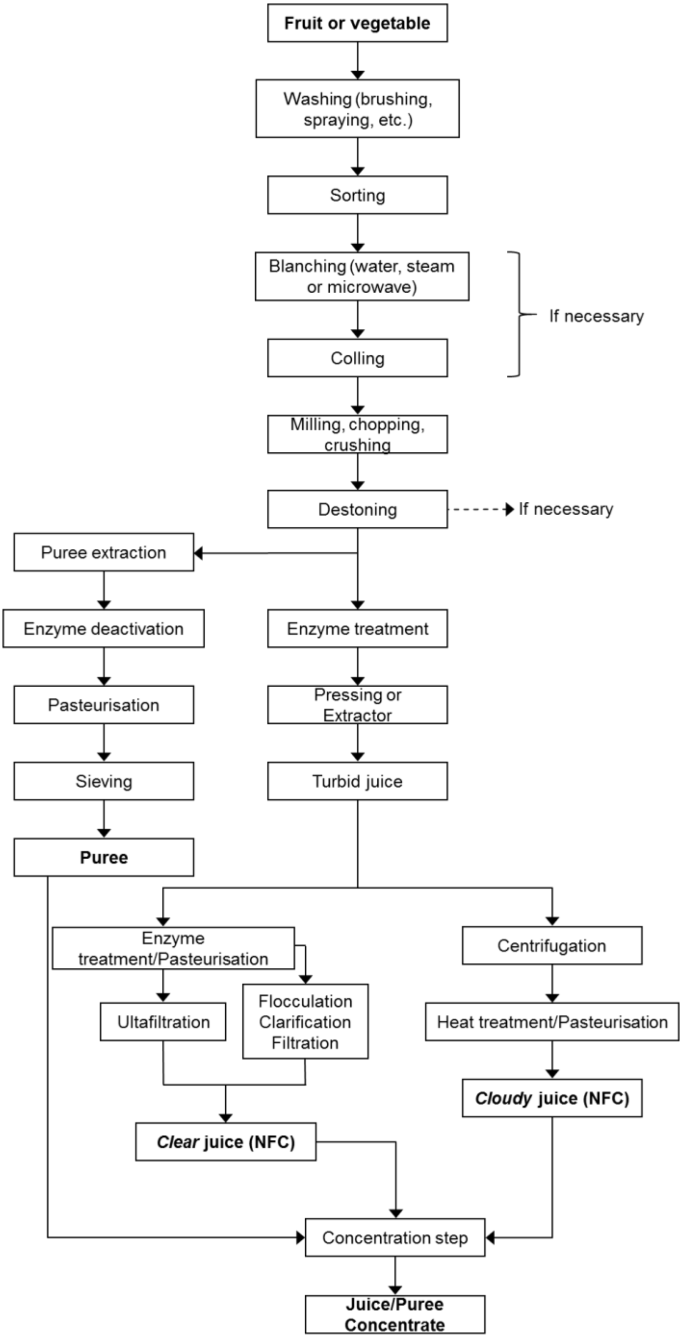


Figure 2. Typical fruit juice (*clear or cloudy)* and puree-processing steps.

Then an enzyme deactivation process is applied and, if necessary, a de-aeration step to prevent the oxidation during heat treatments such as pasteurisation [21]. To increase the shelf life, the puree may then be pasteurised using a controlled time/temperature regime that allows reducing microbiological load whilst maintaining as much of the colour, flavour and aroma of the fruit as possible. Finally, the puree is generally further passed through a sieve (or multiple stages of sieves) to ensure the final product is sufficiently smooth and homogenous [22].

Juice Not From Concentrate.

Juice not from concentrate (NFC) is the fermentable but unfermented product obtained from fruit which is sound and ripe, fresh or preserved by chilling, having the characteristic colour, flavour and taste typical of the juice of the fruit which it comes. No water is added or removed, and process techniques are designed to retain as much of the character of the raw fruit as possible. NFC juices can be categorised by pulp content as *cloudy* or *clear*:

Cloudy: Most juices are naturally cloudy when squeezed/pressed. This is due to fine pulp particles suspended in the product that do not allow light rays to pass through the juice, generating the scattering phenomena [21]. Cloudy juices undergo less processing than their clarified counterparts and so they retain slightly more of the natural character of the fruit from which they are made [20].

Clarified: Clarified juices are processed to remove the fine suspended particles of pulp that naturally give a cloudy appearance to most juices. There are a number of methods by which this can be undertaken, depending on the type of fruit and available processing facilities and in a manner that minimises the impact of the extra processing on the quality of the juice [14].

The general process carried out in a typical fruit juice line (Figure 2) consists in several steps that include washing and sorting of the fruit followed by destoning or deseeding and milling. Next, product is submitted to an enzyme treatment to break down pectin so as to increase the juice yield [23]. The pulp is then passed through a press that extracts the juice by applying physical pressure. In the case of citrus fruits, enzymatic treatment is not necessary and the pressing is replaced by an extractor where the juice is separated from the pulp and skin of the fruit. The obtained juice is cloudy and is then placed in a centrifuge where high speed is used to push the juice through the inner basket and out of the machine whilst retaining any larger particles of pulp and other solids. [24]. If clear juice is required, juice clarifying can be performed by ultrafiltration (UF). UF has been replacing traditional processes, such as the use of finning agents (clarification), filtration additive in press-filters (filtration) and the use of flocking agents (flocculation) [25].

Finally, NFC juice may be pasteurised (sometimes with flash heating) to control the microbiological load of the product in order to improve storage and shelf life.

Concentrated juice and puree.

Concentrated fruit juice is a regulated product. In contrast concentrated puree is defined as a raw material [12]. Concentrated juice and puree are processed to remove a defined proportion of the natural water content found in the fruit ending in a product that is approximately 3 to 7 times more concentrated than the original and, therefore, smaller in volume. Commonly, clear juice concentrates have about 65-70 ºBx, cloudy juice concentrate about 40-60 ºBx and puree concentrate around 16, 20 or 30 ºBx [15, 16]. Concentrates can be stored for longer periods of time and at more convenient temperatures than NFC fruit juices. This can greatly reduce the costs required for transportation of equivalent volumes of concentrate, which can then be returned to its single-strength state by reconstituting with water once it reaches the final processing destination [20].

The common scheme applied to obtain concentrate products includes the same process carried out to obtain the desired processed product followed by the concentration steps [26]. Concentration steps involve the processing of juice through an evaporator, which typically concentrates the product in a series of multisteps. These steps minimise the heat that must be applied to the juice, protecting the colour and flavour of the fruit. Commonly, evaporators operate at low pressure, allowing faster water evaporation at lower temperatures and minimising quality loses during juice processing [27]. Sometimes, the concentrated product is then pasteurised, but this is not necessary for all packaging formats because the evaporator stage applies sufficient heat to control microbiological activity in the product. In this case is necessary to sterilise the filling line and fill drums with steam. As the concentrate is a raw material, it is generally pasteurised when it is reconstituted with water ready to be packed into a consumer format [21].

1.1.2. Canned fruit pulp and vegetables.

Canning is a method of preservation in which a cooked or uncooked food is sealed in tin plated and lacquered steel cans. The first step is washing and sorting of the fruit or vegetables (Figure 3)[28]; the criteria of fruit selection differs, in terms of quality, from that applied to fruit juices and other products related above. Commonly fruit is from selected varieties, which have been harvested and quickly transported to the fruit processing plant. Sometimes, fruits are ripened in controlled ripening chambers or left for natural ripening in open yard [29]. Fruits can be canned in slices, rings, cubes etc. along with sugar syrup of desired brix degrees. Before filling, cans are washed with hot water and sterilised and sometimes these are subjected to a jet of steam to remove dust and foreign material, then an automatic large can-filling machines are used. After filling, the process of removal of air from cans is known as exhausting. There are three methods of exhausting: thermal exhausting, steam flow or steam vac and high speed mechanical vacuum [30]. Immediately after exhausting the cans are sealed, they are sterilised by heat treatment under high pressure retort.

Vegetable canning is similar to fruit canning except that the filling component in this case is a brine solution. They also need specific requirements of processing temperature, time and type of cans [31]. Since vegetables are low in acid they do not usually require lacquered cans.

1.1.3. Dried fruit and vegetables.

Dehydration is among the oldest and most common forms of fruit preservation. In dehydration, moisture in the fruit is driven off; leaving a stable food that has moisture content below that at which microorganisms can grow. There are three basic systems for dehydration: sun drying, such as that used for raisins; hot-air dehydration; and freeze-drying [17].

The advantages of dehydration are various, dehydrated fruit has a virtually unlimited shelf life under proper storage conditions. In addition, dehydrated fruits are typically reduced in weight by 75 to 90 percent and its lack of need of refrigeration, and handling and transportation conditions, allow to reduce the associated cost dramatically. To obtain a convenient dehydrated product form usually requires a careful inactivation of enzymes. This is usually accomplished by blanching of the fruit or by chemical inactivation [32].

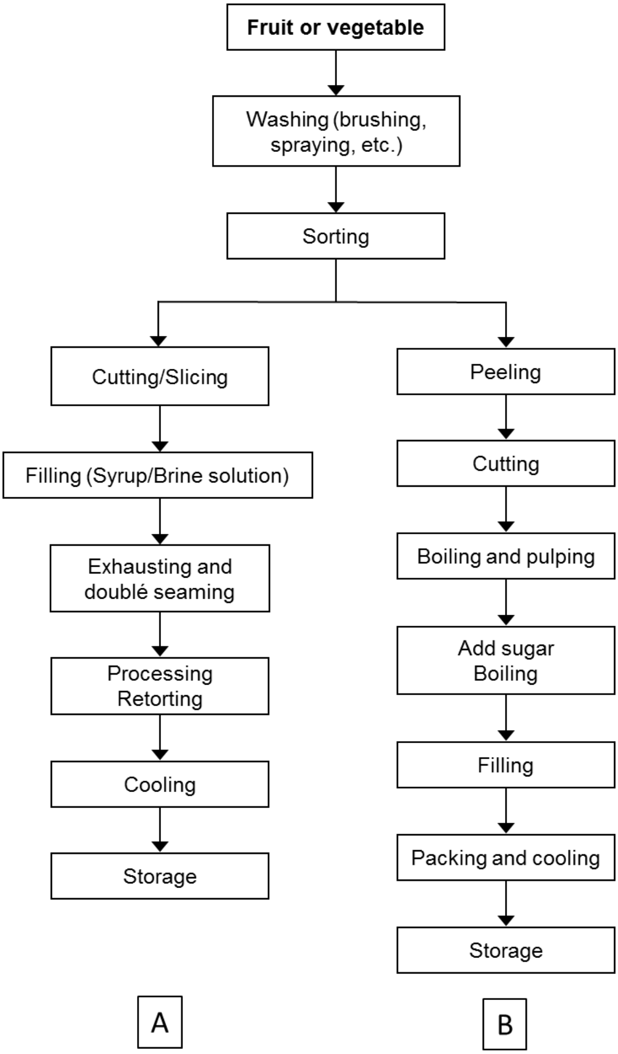


Figure 3. Typical process flow for fruit or vegetable canning (A) and jam making (B).

1.1.4. Fruit and vegetable fibres.

Due to increasing production, waste disposal represents a growing problem since plant material is usually prone to microbial spoilage, thus limiting further exploitation. Therefore, agro-industrial waste is often utilised as feed or as fertilizer. However, demand for feed may be varying and dependent on agricultural yields. The problem of disposing by-products is further aggravated by legal restrictions.

Thus, efficient, inexpensive and environmentally sound utilisation of these materials is becoming more important especially since profitability and expenses may suffer [33].

Fruit and vegetable wastes can be reused because of the health-promoting components and other valuable ingredients that they contain (e.g., carbohydrates, proteins, mineral substances and flavours) [34]. An appropriate fruit and vegetable waste management might considerably reduce the cost of transport and utilisation. Another advantage, in economic terms, is the potential reuse of by-products as raw materials for the manufacture of new products, thus making it possible to reduce the troublesome seasonal pattern from which some industries suffer [35]. Recently, different fibres obtained as fruit and vegetable by-products have been described [35-38]. These fibres, in addition to their physicochemical properties, can also be considered a functional food.

The 'ideal dietary fibre' should meet the following requirements [39]:

1. Have no nutritionally objectionable components.

2. Be as concentrated as possible so that minimum amounts can have a maximum physiological effect.

3. Be bland in taste, colour, texture and odour.

4. Have a balanced composition (insoluble and soluble fractions) and adequate amounts of associated bioactive compounds.

5. Have a good shelf life that does not adversely affect that of the food to be added.

6. Be compatible with food processing.

7. Have the right, positive image to the eyes of the consumer with regard to source, wholesomeness, etc.

8. Have the expected physiological effects.

9. Be reasonable in price.

In this regard, and just as it happens in other processed products from fruit and vegetables, there is a need to develop processes for the preparation of fruit fibres that minimise the losses of associated bioactive compounds (flavonoids, polyphenols, carotenes, etc) which may exert higher health promoting effects than the dietary fibre itself. Wet milling, washing, drying and dry milling are very important in producing fibres from fruits. Drying step should be optimised since this assures the absence of pathogen microorganisms and the possibility to preserve the dried by-products in safe conditions during a long time until their valorisation. It also assures the maximum content of the functional phytochemicals with antioxidant and antimicrobial activity [40]. Figure 4 shows a process flow to obtain food fibres from several fractions coming from juice production lines [16]. This dehydrated production line has stages of homogenisation and milling both pre- and post-drying, and a step of sieving to obtain a homogeneous product as attractive as possible from a commercial point of view.

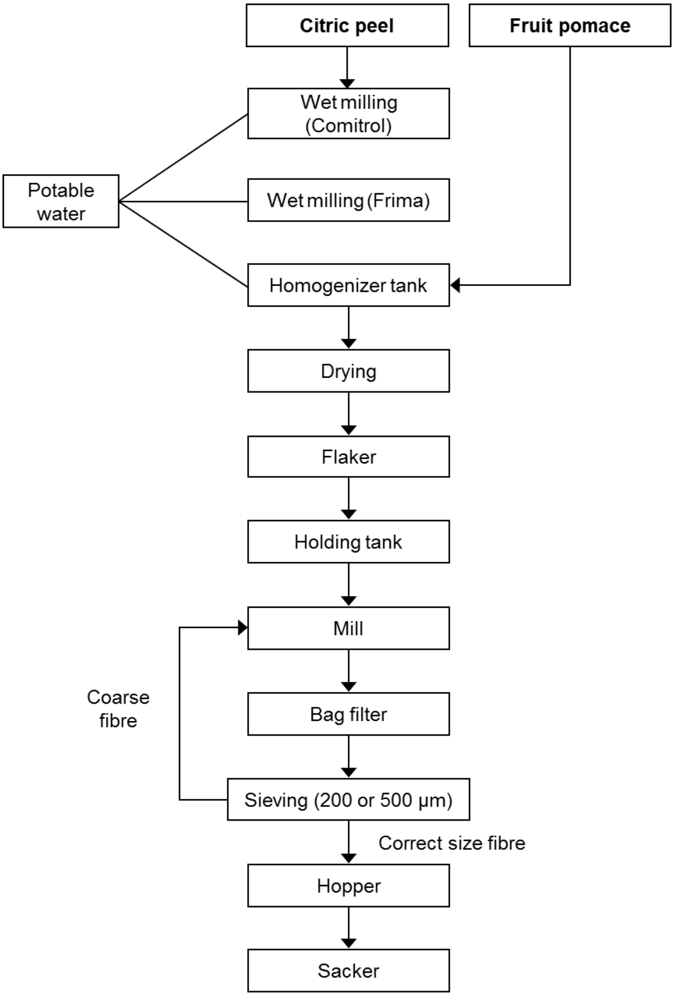


Figure 4. Process flow to obtain fruit or vegetable fibres from juice industry by-products.

1.2. Determination of primary and secondary metabolites of processed fruit and vegetables

1.2.1. Primary metabolites.

Primary metabolites such as carbohydrates, organic acids, amino acids, and fatty acids are involved in plant growth and development, respiration and photosynthesis, and hormone and protein synthesis [41].

1.2.1.1. Carbohydrates, organic acids and amino acids.

Free sugars, organic acids and amino acids are natural constituents of many fruits and vegetables and play an important role in quality attributes and determining nutritive value [41]. The nature and concentration of these constituents in fruits and vegetables are also of interest because of their important organoleptic properties. Free sugars are one of the most important constituents of fruits and vegetables. Monosaccharides and disaccharides, such as fructose, glucose and sucrose, are considered to be the major sugars in most fruits contributing to the flavour of fruits [42]. Organic acids and amino acids and their derivatives also affect the quality of foods including taste, aroma and colour [43]. Fruit sweetness depends on the concentration of the main sugars and can be evaluated based on the following classification relative to sucrose: fructose (1.2) > sucrose (1.0) > glucose (0.64). Similarly, sourness or acidity is dependent on the predominant organic acids. In this case, the degree of acidity of each compound can be classified in relation to citric acid: citric (1.0) > malic (0.9) > tartaric (0.8). Some amino acids, such as aspartic and glutamic, can also be associated with the acidity [44]. Then, sugars, organic acids and amino acids present in food and their relative ratios, can affect the chemical and sensorial characteristics of the matrix (e.g., pH, total acidity, microbial stability, sweetness, global acceptability), which explains the increasing interests in their determination. Although it has been reported in the literature that organic acids [45], sugars [46] and amino acids [47] are not altered during the most common fruit and vegetable industry processes, their analysis can contribute with precious information about food quality or on the optimisation of some selected technological processes. Consequently, accurate identification and determination of organic acids and sugars in fruit juices has to be considered a very important goal since it provides useful information on the nutritional quality, the authenticity of the product and microbiological alteration processes.

Several methods have been published for the analytical determination of sugars, organic acids and amino acids in processed fruit and vegetables. Apart from the differences in structure, which already determine the most appropriate method of analysis, the differences in concentration between families of these kind of products difficult a precise and accurate simultaneous determination of all compound groups. The most commonly used analytical techniques for the determination of sugars and organic acids in processed foods and, in particular, in fruit and vegetable juices, are based either on high performance liquid chromatography (HPLC) [48, 49] or on colorimetric enzymatic measurements [49]. The main detectors coupled to HPLC are refractive index detector (RID) and evaporative light scattering (ELSD) for sugars and ultraviolet (UV) detection at 210 nm for organic acids, although some of them lack of sensitivity and selectivity. In contrast, mass spectrometry presents high sensitivity and selectivity, but has high analytical cost and has a narrower linear range. Regarding to amino acids determination in fruit and vegetable products, the analytical methods used are mainly based in HPLC with photodiode array (PDA) and fluorescence detection (FLD), including pre or post-column derivatisation (e.g., O-phthaldialdehyde -OPA-, ninhydrin, phenylisothiocianate -PITC-, fluorescamine, 9-fluorenylmethyl chloroformate -FMOC-, …) [50, 51], others based in mass spectrometry detection with pre-column derivatisation (e.g, dansyl chloride -DNS-, diethyl ethoxymethylenemalonate -DEEMM-, p-N,N,N-trimethylammonioanilyl N'-hydroxysuccinimidyl carbamate iodide -TAHS-, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate -AQC-, …) [52] and others based in using non reversed-phase chromatography [53, 54]. On the other hand, it has been published the simultaneous identification and quantitation of sugars, sugar alcohols, carboxylic and amino acids by gas chromatography (GC) coupled to mass spectrometry (MS) from a single solution, in one single injection, as their trimethylsilyl ether/ester derivative (TMS) in the case of free hydroxyl and carboxyl groups, and the corresponding oxime derivative in the case of the carbonyl group of sugars [55-58]. In the case of matrices containing constituents with various functional groups as in the case of the samples included in this study, the GC-MS of their TMS/oxime ether/ester derivatives is the method of choice [55, 56]. In terms of MS, the common ionisation source applied is electron ionisation (EI), also formerly known as electron impact ionisation. Despite the high fragmentation produced by EI, which difficult or avoids the molecular ion, the spectra observed under standard conditions are reproducible and independent of the instrument used. This feature has allowed to create extensive available spectral libraries, which include chemical derivatised compounds (e.g, amino acids, organic acids and sugars) and facilitates their identification without standards. In addition, studies on the fragmentation patterns of various homologous series of acids [59], as well as on the well-defined and characteristic proportions of oximes from reducing sugars, allows their separation and determination in the presence of each other. This has been achieved even at largely different concentrations of both groups of compounds in fruits and vegetables and also in their processed products such as orange juice [60, 61] or quince juice [62]. Despite the clear advantages, it should also be noted that it involves a long sample preparation process and the introduction of variability through derivatisation. In addition, problems related to steric hindrance might lead to mixtures of fully and partially derivatised analytes and the analyst should be aware of the possible formation of artefacts during the trimethylsilylation process.

Though less sensitive than HPLC or GC, nuclear magnetic resonance (NMR) has several advantages. It is nondestructive, selective and capable to perform the simultaneous detection of a great number of low molecular mass components in complex mixtures. Moreover, sample preparation is easy to carry out and less time consuming. Solid-state NMR and ^{1}H-NMR relaxometry [63, 64] have been largely discussed, and several applications can be found in literature. In solid-state NMR spectroscopy, high-resolution magic angle spinning (HR-MAS) has recently been successfully used in foodstuff characterisation, in particular of fruits and vegetables. HR-MAS offers an almost unique opportunity to obtain highly resolved NMR spectra without any chemical, physical and/or biological manipulation of the sample. Nevertheless, high-resolution NMR spectroscopy of liquid extracts remains the most common approach. [65, 66]. Metabolite identification is usually obtained by means of 1D and 2D experiments [67], spiking experiments (i.e., addition of standard compounds to the analysed sample), literature data and by comparison with databases of standard compounds.

Several works have suggested ^{1}H-NMR analysis for the identification as well as for quality control of fruit and vegetable juices. Several classes of compounds ranging from main sugars to minor compounds such as amino acids are observable in the ^{1}H-NMR spectra of fruit juices (Figure 5). ^{1}H-NMR allows two kinds of analysis of fruit and vegetable juices within one experiment: namely targeted and non-targeted determination. In the first one, signals are previously selected, based on descriptions in the literature, in order to quantify the compound. So far, this approach has been detailed for different fruits such as apple [68-70], peach [71], pear [72], orange[73, 74], grape [73, 75], mango [76-78], kiwi [79] and tomato [80, 81] and vegetables as lettuce [82], onion [83], sweet pepper [84] and carrot [85]. Literature provides absolute quantification examples of the major metabolites including soluble sugars [85], organic acids [86, 87], amino acids [88] and some major secondary compounds [80, 81]. In contrast, the non-targeted determination uses all spectrum signals and employs multivariate statistical analysis to build a model to predict facts as if a juice has been adulterated or to foresee its geographical origin. In this sense, it is interesting to highlight that a novel ^{1}H-NMR analyser was commercialised under the trade name "JuiceScreener" [89, 90], that combines statistical modelling with molecule specific signal analysis making it a powerful tool for food quality assessment. Combining both, targeted and non-targeted analysis, Juice Screener simultaneously evaluates, within a few minutes, a large number of quality-related parameters (e.g., for apple juice, it allows the quantification of 24 substances and provides 4 calculated values (i.e., glucose/fructose ratio, malic acid/quinic acid ratio, % sucrose, and total sugar) as well as some authenticity-related parameters (e.g., type of juice and geographical origin). Whilst this has advantages, especially as the operator needs only minimal training, the implementation of underlying algorithms and data validation is opaque to the user. There is current research to validate the NMR JuiceScreener methods for analysing different juice matrices (fruit types) in order that the method may be applicable for quantitative juice measurements in official food control (regulation (EC) No. 882/200).

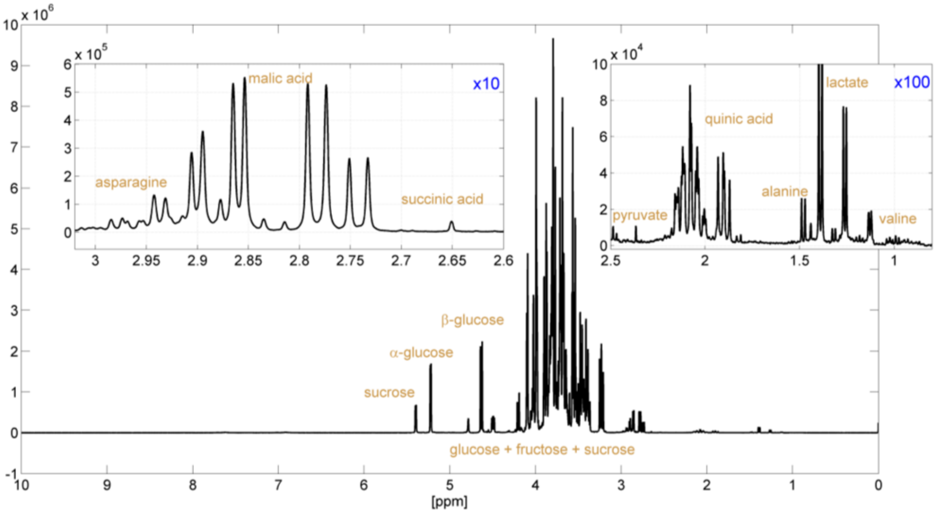


Figure 5. ^{1}H-NMR spectra of an apple juice and two enlargements showing signal assignments of a few key compounds. The x-axis corresponds to the chemical shifts of the proton resonances, which are dependent on the chemical environment of the protons (e.g., type of bonds and neighbouring atoms). The y-value is linearly dependent on the molarity of the corresponding proton(s) [90].

1.2.1.2. Fatty acids.

The edible part of fruits and vegetables generally contains very low levels of lipid material (0.1%–1.0%) and as such, they do not constitute an important source of edible or industrial fats and oils [91]. A few notable exceptions are nuts, avocado, palm, and olive. Nevertheless, even a small amount of lipids present in a food product makes it more palatable and satisfying, facilitates the utilisation of proteins and fat-soluble vitamins and serves as the structural material of cell membranes in all tissues [92]. Each fatty acid plays a significant role in normal biological functions and in relation to major problems in health and agriculture. The type and quantity of fatty acids as well as the position and distribution of these fatty acids in the triacylglycerol molecule determine their chemical, physical, and functional properties [93]. The knowledge of the fatty acid composition may help to facilitate new food product development. During the processing of fruits and vegetables, several types of oxidative reactions may occur leading to the formation of an oxidised form. These reactions cause browning reactions, loss or changes to flavour or odour, changes in texture and loss of nutritional value due to the destruction of vitamins and essential fatty acids. These changes are important in fruits and vegetables. Especial problems arise in seed crops and lipid-containing vegetables leading to the development of rancid flavours and oxidation products that may have toxic properties at high levels [94]. On the other hand, the processing of many fruits results in the accumulation of large quantities of seeds and kernels. Proper utilisation of these by-products could reduce waste disposal problems and serve as a potential new source of fats and proteins to be used in food and feed. The lipid content in seeds can be high, ranging in most cases between 20% and 60%. Moreover, most oils obtained from fruit seeds have a greater percentage of unsaturated than saturated fatty acids [91] increasing the capability of oxidative reactions.

Analysis of fatty acids (FAs) is always preceded by a lipid extraction normally using a solvent system. Various alternatives have been reported; such as Chloroform/methanol (2:1, v/v) [95], Chloroform/methanol (1:2, v/v) [96], Hexane/isopropanol (3:2, v/v) [97], Chloroform/isopropanol (7:11, v/v) [98] and Chloroform/methanol/12 N HCl (2:4:0.1, v/v/v) [99]. Analysis of FAs becomes even more complex because, although they can occur in nature as free FAs, they are usually found in the form of esters linked to glycerol (glycerolipids defined as monoacylglycerols -MAGs-, diacylglycerols -DAGs- and triacylglycerols –TAGs- and their derivatives), long chain aliphatic alcohols, and amides as sphingolipids. Liquid chromatography (LC) coupled to MS or tandem mass spectrometry

(MS/MS) (LC-MS or LC-MS/MS) with soft ionisation such as electrospray ionisation (ESI), that can generate the intact species such as $[M+H]^+$ or $[M+NH_4]^+$, have mainly been used for the structural characterisation of different classes of glycerolipids. The separation of neutral lipids has successfully been performed using normal-phase (NP) columns based on thin-layer chromatography which has also been extensively used to isolate these compounds. Also, LC coupled with MS has been used for the separation and characterisation of phospholipids or sphingolipids. Despite the great information about the composition of FAs given by LC-MS technique, the robustness and precision of GC with flame ionisation detection (FID) should not be underestimated. FID is robust and has a high dynamic range, allowing an accurate quantification. Fatty acid methyl esters (FAMEs) are used almost universally for GC analysis of fatty acids. The fatty acid chain is converted to low molecular weight nonpolar derivatives. To prepare methyl and other esters of FAs , it is not necessary to previously release the free FAs acids from the lipids by hydrolysis as they can be directly transesterified [100, 101]. FAs, the major constituents of most of the lipid components present in foods, are then converted into FAMEs and analysed by GC/FID. Because of the huge structural variety of FAs (chain lengths from 4 to 28 carbons, 0-6 double bonds including *cis* and *trans* isomers, alkyl branches, and functional groups), co-elution is inevitable on virtually all GC stationary phases. Therefore, to assert the fatty acid identification, two columns with different chromatographic phase are used in many cases. For these reasons, GC coupled to EI-MS analysis in the full scan and selected ion monitoring (SIM) modes have been used for verification of the results obtained by GC/FID. Because of the higher selectivity of this detection method, co-eluting pairs of FAs can be identified [37]. Recently, comprehensive two dimensional gas chromatography (GC × GC) has arisen as an interesting complementary technique to analyse fatty acids from different sources [37]. The few studies published on fruit and vegetable fatty acids analysis have been performed with GC coupled to FID. Fatty acid profiles for fruits and vegetables are mainly composed of unsaturated fatty acids, specifically oleic (18:1), linoleic (18:2) and linolenic (18:3) [102]. Regarding fruit juices, Arena et al. studied the composition of FAs in two types of Italian blood orange juices, one NFC and another reconstituted from concentrate (RFC). Significant differences in fatty acid distribution have been found in the isolated fractions, but each class exhibits similar profiles both in NFC and in RFC juices, indicating that no selective degradation of any acid occurs during the process of thermal evaporation. Four fatty acids (linoleic, palmitic, linolenic, and vaccenic) constitute 88% of total fatty acids [103].

1.2.1.3. Chlorophyll and derivatives.

Chlorophyll is an extremely important biomolecule, critical in photosynthesis, which allows plants to absorb energy from light. Chlorophyll molecules are specifically arranged around photosystems that are embedded in the thylakoid membranes of chloroplasts. The function of most chlorophylls is to absorb light and transfer that light energy to reaction centres [104]. Chlorophyll and its derivatives have a long-established history of use in medical applications. Natural chlorophyll and commercial grade derivatives such as sodium copper chlorophyllin (SCC) have been widely investigated for a range of beneficial biological activities, such as anti-inflammatory, wound healing, internal deodorisation [105] and recently described antioxidant and antimutagenic activities has attracted an increasing number of studies as a tool for preventing human cancers [106]. Chlorophyll is a tetrapyrrole based compound which structure consists of a porphyrin ring coordinated to a central atom and a long phytol group in the tail. This structure is similar to the heme group of hemoglobin, but with a central magnesium in place of iron atom [107]. There are actually two main types of chlorophyll, named *a* and *b,* (Figure 6).

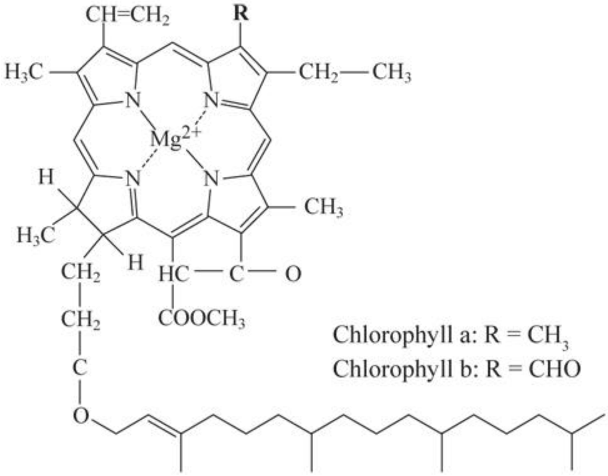


Figure 6. Basic chemical structure of chlorophylls *a* and *b.*

Chlorophyll *a* is the primary photosynthetic pigment in plants, whilst chlorophyll *b* is an accessory pigment that acts indirectly in photosynthesis by transferring energy to chlorophyll *a*. They only differ slightly, in the composition of a side chain (a -CH_3 group in *a* is, a CHO group in *b*), translating these structural differences in different pigmentation. Whilst chlorophyll *a* has a blue/green coloration with a maximum absorbance at 660-665 nm, chlorophyll *b* has its maximum between 642-652 nm and green/yellow colour [108].

Chlorophyll is the pigment responsible for the green colour in fruit and vegetables, which is an indicator of the quality and attractiveness of these to consumers. High levels of chlorophyll have been described in fruits such as kiwi, avocado, pear and apple skins and in vegetables such as broccoli, spinach, zucchini, etc [109, 110]. Other foods also considered as very rich sources of chlorophyll have been algae and teas [111, 112]. The decomposition of chlorophylls in foods and the analysis of chlorophyll derivatives have been the subject of numerous investigations because of the importance of these pigments in the maintenance and quality of colour in fruit and vegetable products. It has been stated that chlorophylls are susceptible to chemical changes during processing, arising different chlorophyll derivatives. Processing leads to acid release that provokes the central magnesium atom replacement by hydrogen ions, rendering a chlorophyll derivative referred as pheophytin [113, 114]. During processing, endogenous enzymatic activity can also induce dephytilation of chlorophyll , mainly rendering pheophorbide, and chlorophyllides under high pH conditions [115] (Figure 7). Demetalitation and epimerisation are also promoted during processing and an additional decarbomethoxylation can happen, mainly originating pyropheophytin. The loss of green color due to the loss of magnesium from the porphyrin ring produces a loss of attractiveness of the product to the consumer. In this context, the formation of metallo-chlorophyll complexes has been studied to re-green processed vegetable and fruit products. Copper and zinc are the metals mainly inserted in the porphyrin ring. The metallo-chlorophyll complexes are more stable than the naturally occurring magnesium one. In this way several green food colorants are reported and regulated in the list of food additives of European Union [116]. Within the green colorants, E-140 is a chlorophyll extract commonly obtained by organic solvent extraction of nettles, spinach and grass. E-141 is obtained by the addition of copper to the extract of E-140. E-141(i) and E-141 (ii) are hydrosoluble and liposoluble copper complexes of chlorophylls (Cu-chlorophylls) or chlorophyllins (Cu-chlorophyllins), respectively. The solvents authorised in the extraction process are acetone, dichloromethane (DCM), methanol, ethanol, propanol and hexane [117].

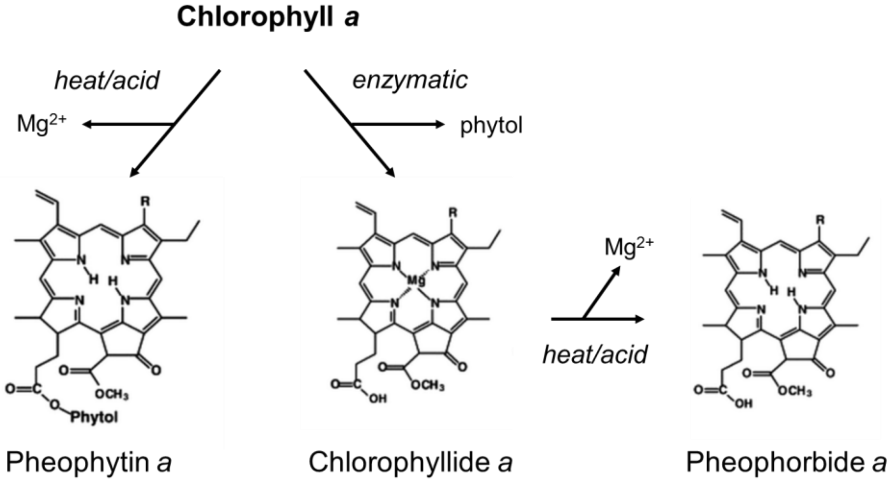


Figure 7. Chlorophyll *a* and its degradation products after heat treatment and/or enzymatic treatment [118].

The analysis of chlorophylls involves various stages, including an extraction step prior the quantitation of the extract via ultraviolet-visible (UV-Vis) spectroscopy or LC coupled with different detectors. Due to the lipophilic nature of these compounds solvents such as methanol, acetone and dimethylformamide (DMF) are commonly used in the extraction as they are water miscible which is adequate for fresh plant tissue. They break the linkage between chlorophyll and protein and extract the pigment. Sometimes a liquid-liquid partitioning step is included in the extraction, using non water soluble solvents such as diethyl ether, ethyl acetate or petroleum ether to transfer the chlorophylls and their derivatives [119]. This step is not recommended for more polar derivatives, since they could be poorly recovered. Considering the above discussed effects of pH on chlorophylls, it is important to maintain a neutral pH during the extraction in order to avoid demetalitation or dephytilation processes. To prevent it, sometimes $MgCO_3$ is added or the extraction solvent is buffered with compounds such as phosphate [120], 2-(N-morpholino)ethanesulfonic acid (MES)[119] and Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffers[109]. In spectrophotometric analysis, the Folch method has mainly been applied to quantify chlorophylls, which consists in an extraction with a 2:1 chloroform-methanol (v/v) mixture followed by a phase separation favoured with addition of NaCl water solution. [121-123].

Chromatographic analyses of chlorophyll and their derivatives in extracts of fruit and vegetables and their processed products are usually performed by HPLC and requires solvents compatible with their lipophilic characteristics. The reversed-phase (RP) C18 columns have been the most used according to the literature [112, 124-126], although there are several publications that use C30 packed columns [127]. The ultra-performance liquid chromatography (UPLC) technology has also been applied in the separation of chlorophylls [128-130], although it should be noted that in none of the publications a full separation of all chlorophyll derivatives is achieved. To achieve the chromatographic separation for the most apolar chlorophyll derivatives it is necessary to use organic solvents, commonly acetone [124, 125]. When the mixture contains dephytilated chlorophyll derivatives, more apolar solvents are avoided as is related below. Due to the acidity ($2<pKa<4$) and high polarity of the chlorophyll derivatives obtained from dephytilisation (chlorophyllides and pheophorbides) simultaneous analysis with apolar chlorophyll derivatives is difficult, [131]. This analysis needs the use of water in the mobile phase and also reagents for ion pair formation. The commonly used buffer as ion-pairing reagent is ammonium acetate. It provides excellent separations for the more polar chlorophyll derivatives as well as several related compounds including the aforementioned green colorants [120, 132]. HPLC coupled to PDA has been the most common technique used for quantitative and qualitative analysis of chlorophyll derivatives. The use of MS for structure elucidation of chlorophyll derivatives using single quadrupole [125, 126], triple quadrupole (QQQ) [130, 133] and quadrupole-time of flight (Q-TOF) [134] is also described in the literature. Ionisation sources as atmospheric pressure chemical ionisation (APCI) [125, 126, 134] and ESI [135, 136] have been used coupled to chromatography. Several papers have studied the analysis of chlorophyll and their derivatives in olives and oils, including various types of processing [132, 137, 138]. These compounds have been studied through RP chromatography in plant samples such as spinach [139], pear skins [140], culinary herbs [141] and teas [111].

1.2.2. Secondary metabolites.

Plants produce a very diverse set of organic molecules, some of which are not traditionally considered to participate directly in the major processes involved in growth and development. According to the nomenclature adopted by the British Nutrition Foundation, plant secondary metabolites can be divided into four major groups: phenolic compounds, terpenoids, alkaloids, and sulphur containing compounds [142]. Unlike primary metabolites, the so-called secondary metabolites are often unevenly distributed among taxonomic groups within the plant kingdom.

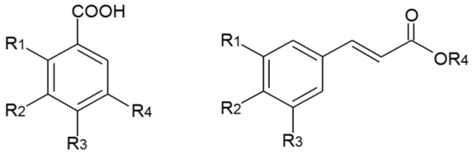
1.2.2.1. Phenolic compounds.

Phenolic compounds are secondary plant metabolites which, as bioactive chemicals, play an important role in plant's defensive arsenal. These compounds provide an efficient protection against pathogens and predators due to their bioactive properties as attractants for pollinators, antifeedants, phytoalexins, antioxidants and protective agents against UV light, amongst others [143, 144]. They are also known for their benefits on human health: antioxidant, antiviral, antiallergenic activities have been described so as anticancer and cardioprotective effects. They are important determinants in the sensory and nutritional quality of fruits, vegetables and other plants and processed products obtained from them. Besides, some contribute to the colour of food [145, 146].

According to the literature, there are two large groups of phenolic compounds based on the extraction procedure needed for their analysis. A first group includes all those phenolic compounds that can be extracted with aqueous-organic solvent and a second group includes all those that require chemical or enzymatic hydrolisation of the food matrix to release polyphenols before the chromatographic or spectrophotometric analysis [147]. Several databases on phenolic compound contents in foods have been developed in the literature. These include the developed by the USDA [148], EuroFIR-BASIS [149] and Explorer Phenol by INRA, the NUTRIALIS program of the French Ministry of Research (2002-2004) and by Unilever, Danone and Nestle (2005-2009) [150, 151]. In these databases phenolic compounds are described in families which are: phenolic acids, flavonoids, tannins and stilbenes and lignans.

Phenolic acids constitute about one-third of the dietary phenols, which may be present in plants in free and bound forms [147]. Bound-phenolics may be linked to various plant components through ester, ether, or acetal bonds [152]. Phenolic acids consist of two subgroups, the hydroxybenzoic and hydroxycinnamic acids. Hydroxybenzoic acids include gallic, p-hydroxybenzoic, protocatechuic, vanillic and syringic acids, which have a common C6–C1 structure (Figure 8). Hydroxycinnamic acids, on the other hand, are aromatic compounds with a three-carbon side chain (C6–C3), caffeic, ferulic, p-coumaric and sinapic acids being the most common representatives [153] (Figure 8). Hydroxycinnamoylquinic acids have been found in high contents in fruit and vegetables such as apple, peach, pear, carrot, etc, including 3-*O*-Caffeoylquinic acid, more commonly known as chlorogenic acid.

Benzoic acid derivatives	**R_1**	**R_2**	**R_3**	**R_4**
Salicylic acid	OH	H	H	H
Gentisic acid	H	OH	H	H
p-Hydroxybenzoic acid	H	H	OH	H
Protochatechuic acid	H	H	OH	OH
Vanillic acid	H	H	OH	OCH_3
Gallic acid	H	OH	OH	OH
Syringic acid	H	OCH_3	OH	OCH_3



Cinnamic acid derivatives	**R_1**	**R_2**	**R_3**	**R_4**
Caffeic acid	OH	OH	H	H
Ferulic acid	OCH_3	OH	H	H
Sinapic acid	OCH_3	OH	OCH_3	H
p-Coumaric acid	H	OH	H	H
Chlorogenic acid	H	OH	OH	$C_7H_9O_5$

Figure 8. Main classes of benzoic and phenolic acids and chemical structures of the cinnamic acid derivatives.

Flavonoids, are formed via condensation of a phenylpropane (C6–C3) compound with the participation of three molecules of malonyl coenzyme A, which lead to the formation of chalcones that subsequently cyclise under acidic conditions. Chemically, they have the general structure of a 15-carbon skeleton, which consists of two phenyl rings (A and B) and a heterocyclic ring (C) (Figure 9). This carbon structure can be abbreviated C6-C3-C6 [154].

Flavonoids can occur as aglycones in glycosides and methylated derivatives. Different patterns of substitution on the ring C result in different classes of flavonoids, among which are: flavonols, flavones, flavanones, flavanols (or catechins), isoflavones, flavononols, and anthocyanidins. Substitutions to rings A and B give rise to different compounds within each class of flavonoids. Within each class, individual flavonoids may vary in the number and distribution of hydroxyl groups as well as in their degree of alkylation or glycosylation. Flavones and flavonols are the main flavonoids in fruits and vegetables. These compounds possess a double bond between C-2 and C-3. The difference between flavonols and flavones is that flavonols possess a hydroxyl group in the 3- position. The main flavonol aglycones in fruit and vegetables are quercetin, kaempferol, myricetin and isoharmnetin and the main flavone aglycones are apigenin, luteolin and diosmetin. All these compounds have a hydroxyl group in the in C-5 and C-7 positions of A

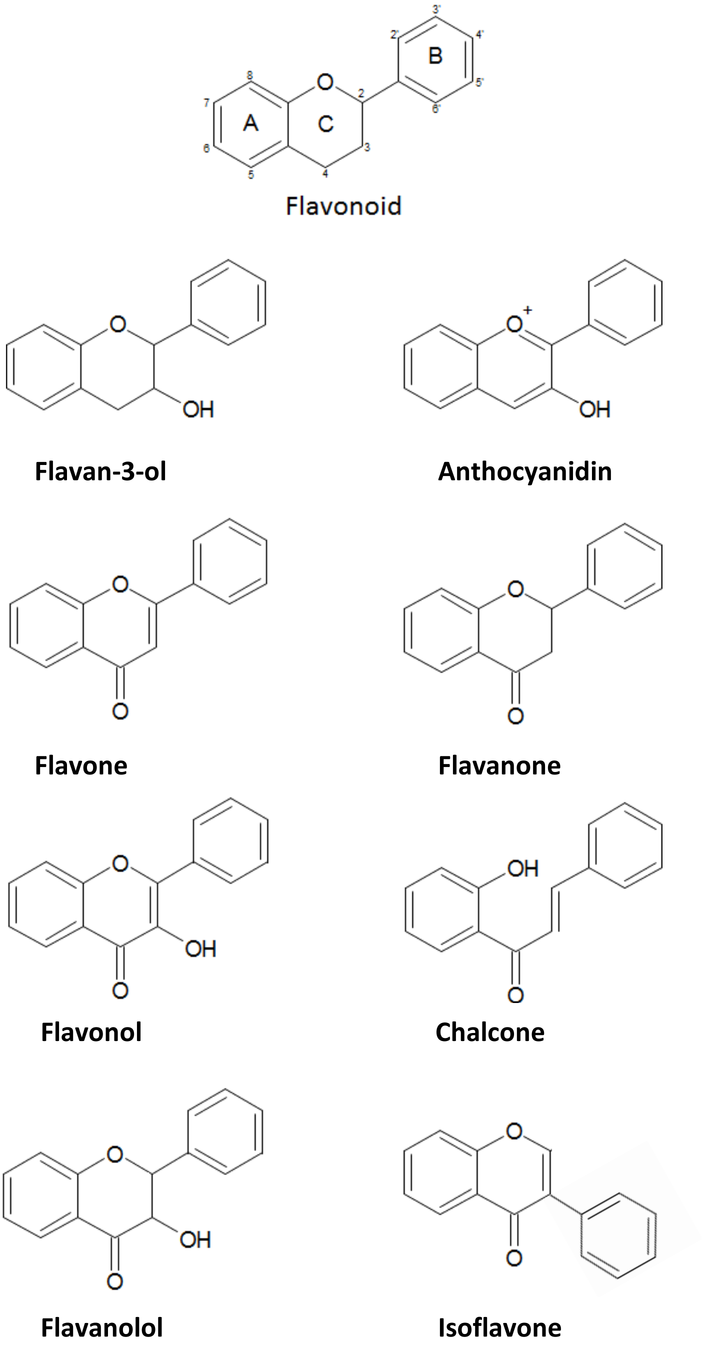


Figure 9. Basic chemical structures of the most common flavonoid subclasses.

ring and differences among them are given by the substitution on C-3', C-4' and C-5' in ring B. Flavonols and flavones are present mainly as mono-, di- and triglycosides. The sugar moiety of glycosides is usually composed of D-glucose, D-galactose, L-rhamnose, L-arabinose, D-apiose and D-glucuronic acid or their combinations. Flavonols are commonly found in fruit and vegetables such as apple, pear, peach, nectarine, cranberries, lettuce, beans etc. On the other hand, flavones are found in citrus fruits, parsley, celery, spinach, etc. [154].

Flavanones are characterised by the presence of a 4H-1-benzopyran-4-one ring. They are generally glycosylated by a disaccharide in C7. Flavanones are present in high concentrations only in citrus fruits, but they are also found in tomatoes and certain aromatic plants such as mint. The main aglycones are naringenin, hesperetin, isosakuratenin in citrus fruit, and eriodictyol mainly in lemons. Flavanones are the most important flavonoids without substituents in C-3.

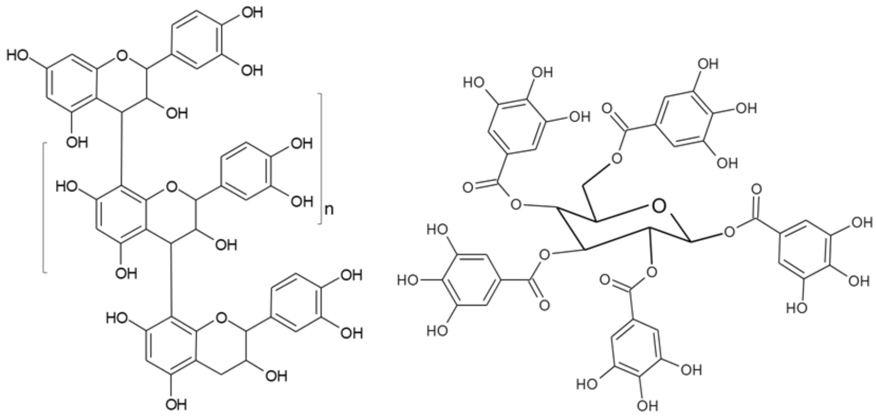
Anthocyanidins, the sugar-free counterparts of anthocyanins, are common plant pigments. They are characterised for containing in its structure the flavylium ion or 2-phenylchromenylium in salt form, which is a type of oxonium ion (chromenylium is referred also to as benzopyrylium). They form a large group of polymethine dyes. The counterion of the flavylium cation is mostly chloride. Anthocyanidins differ from other flavonoids by their positive charge. Bilberries, black and red currants, blueberries, cherries, chokecherries, grapes, strawberries are fruits where these pigmente are present.

Flavan-3-ols are derivatives of flavans that use the 2-phenyl-3,4-dihydro-2H-chromen-3-ol skeleton and include catechin, epicatechin gallate, epigallocatechin, epigallocatechin, gallate, proanthocyanidins, theaflavins, thearubigins. They are found in fruits like apple, pear, peach, blueberries and lettuce etc.

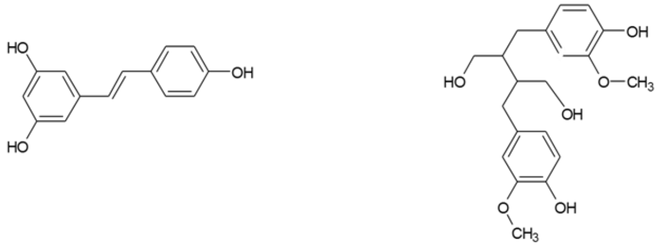
Tannins are relatively high molecular weight compounds which constitute the third important group of phenolics. It may be subdivided into hydrolysable and condensed tannins (Figure 10) [155]. One particular type of condensed tannins, found in several fruits, are procyanidins, which are polymers of 2 to 50 (or more) flavan-3-ol units joined by carbon-carbon bonds. Hydrolysable tannins are derivatives of gallic acid (3,4,5 trihydroxyl benzoic acid) [156].

Another group of phenolic compounds are the stilbene derivatives which are present in low quantities in the human diet. The main representative of this group is resveratrol (Figure 10), that exists in both *cis* and *trans* isomeric forms, mostly in glycosylated forms [157].

Finally, lignans are produced by oxidative dimerisation of two phenylpropane units (Figure 10); they are mostly present in nature in the free form, whilst their glycoside derivatives are only a minor form.



Condensed tannin **Hydrolisable tannin**



Resveratrol **Secoisolariciresinol**

Figure 10. Scheme of condensed and hydrolysable tannins and structures of the resveratrol stilbene and the secoisolariciresinol lignan.

The effect of fruit and vegetable processing on phenolic compounds has been studied in different publications. Fruit juices as grapefruit, orange or apple are abundant sources of natural phenolic compounds [158, 159]. Researchers have a common aim to find the best conditions and processing techniques to improve the health-promoting and nutritional properties of juices. Furthermore, the influence of each current processing technique (pasteurisation, freezing, and concentration) on phenolic compounds, vitamin C, and antioxidant capacity has been described at pilot or experimental plant level and at industrial scale [160]. Numerous authors have shown that production time and processing steps, especially during heat treatment, degrade phenolic compounds and vitamin C, and decrease the nutritional quality and antioxidant properties of juices [161]. According to Hernandez et al. thermal treatments applied to peach and apple juices affected their phenolic composition [159]. The effect of pasteurisation on total phenol reduction was evaluated by Alper et al. [158] finding a reduction about 20 %. In orange pulp, pasteurisation led to degradation of several phenolic compounds such as caffeic acid derivatives, vicenin 2 (apigenin 6,8-di-*C*-glucoside), and narirutin (5,7,4'-trihydroxyflavanone-7-rutinoside), with losses of 35, 30, and 28%, respectively [160]. In the case of proanthocyanidins a decrease of 10% has been disclosed after the thermal treatment in peach juice production [162]. During orange juice storage, the obtained results indicate that vitamin C and free and total phenolic acids were affected by storage time and temperature. In contrast, small changes in flavanone content were observed indicating a high stability of these compounds upon storage [163]. Rababah et al. [164] found a reduction around 95 % in phenolic compounds after jam processing. In spite of such reduction in these valuable compounds, jam retained good amounts of them during storage. By-products remaining after processing of fruits and vegetables still contain a huge amount of phenolic compounds. In the same way, fibres obtained as by-products from fruit and vegetable processing have been described as a good source of phenolic compounds [39]. Total phenolic content has been studied in by-products and fibres obtained after processing various types of fruit and vegetables, such as apple, peach, pear, citrus fruits, carrot, and spinach [36, 38, 49, 165, 166, 167].

Extraction is a very important step in the analysis of phenolic compounds in processed fruit and vegetables and there is no single and standard extraction methodology. Hence, the optimisation of the extraction procedure is essential for an accurate assay of phenolic compounds from different matrices. Solvents, such as methanol, ethanol, propanol, acetone, ethyl acetate, DMF and their combinations have been used for the extraction of phenolic compounds, often mixed with different proportions of water [168]. It is difficult to perform a unique method for the optimal extraction of all phenolic compounds due to the significant variations in their polarities. Procedures based on extraction sequences are commonly applied using different solvents to extract the maximum of phenolic compounds. To improve the extraction process, alternative modern extraction and isolation techniques have been described such as supercritical fluid extraction (SFE) [169], pressurised liquid extraction (PLE) [170], microwave-assisted extraction (MAE)

[171] and ultrasound-assisted extraction (UAE) [172]. On the other hand, bound phenolic acids need a previous base or acid (or both) hydrolysis to be released from the sugar moiety [173, 174].

The analysis of phenolic compounds can be classified within assays that determine the total phenolic content (TPC), or those that identify and quantify a specific family or type of phenolic compounds. Folin-Cicolteau assay is widely used for the determination of total phenolic content in several processed fruit and vegetable products [175]. This assay is a colorimetric method based on electron transfer reactions between the Folin-Cicolteau reagent and phenolic compounds. However, this assay is not specific for TPC determination. Other compounds that are present in food extracts such as aminoacids and reducing sugars have also been described to reduce the reagent so interfering TPC results [176]. Several alternatives have been proposed to improve this methodology including higher purification through solid phase extraction (SPE) or the application of chemometric methods that allow the creation of models to correct the obtained overestimation.

The most widely used technique to analyse particular phenolic compounds is LC. For chromatographic separation, different columns and mobile phases are used depending on the group of compounds to separate. HPLC has been widely used during the last two decades, however, in recent years, UPLC has arisen. UPLC allows an improvement in the separation and the limits of detection and reduces the time of analysis. Chromatographic columns with fused core technology have also been introduced with results similar to UPLC separation but with HPLC conditions. Thus, to separate unbounded polyphenols such as flavan-3-ols, flavonoids and hydroxycinnamic acids, a chromatographic system consisting of a RP column (C18 or variants thereof) is often used eluting with a mobile phase composed of acetonitrile and water (slightly acidified) [177]. In contrast, for the separation of anthocyanidins, mobile phases are strongly acidified in order to have the compounds in neutral form to be retained in the chromatographic column [178]. For the separation of proanthocyanidins NP columns are usually used, because they allow separations according to their degree of polymerisation.

Plant phenolic compounds are commonly detected using UV–Vis detectors, as PDA. Other methods used for the detection of phenolics include electrochemical coulometric array (EC) detector, chemical reaction detection technique and FLD [179, 180]. As MS is particularly useful for identification, this technique has also been used as a method for the detection of phenolic compounds in processed fruit and vegetables. The main ionisation sources used to analyse phenolic compounds are: fast atom bombardment (FAB) [181], atmospheric pressure ionisation (API) including, ESI [182, 183], APCI [184, 185], atmospheric pressure photo-ionisation (APPI) [186] and matrix-assisted laser desorption ionisation (MALDI) [187, 188]. The MS/MS approach is a very powerful tool that allows flavonoid aglycone and sugar moiety characterisation. This information may be obtained from fragmentation at source, QQQ [182, 183], Q-TOF [189, 190] and ion trap (IT) [191]. Thus, many publications have appeared describing the fragmentation patterns of the various phenolic compounds in order to achieve identifications without the need for direct comparison with pure standards. GC-MS, after hydrolysis and derivatisation, as well as approaches with less sensitive techniques such as near infrared spectroscopy (NIR) [192, 193] and NMR [194, 195] that need previous concentration of the samples, have also been described for the analysis of phenolic compounds.

1.2.2.2. Carotenoids.

Carotenoids are localised in subcellular organelles (plastids), *i.e.* chloroplasts and chromoplasts. In chloroplasts, they are mainly associated with proteins and serve as accessory pigments in photosynthesis, whereas in chromoplasts they are deposited in crystalline form or as oily droplets [196]. In plants, where chlorophyll is present, some of the carotenoids such as xanthophylls are involved in photosynthesis taking part in energy transfer. Many dietary fruits and vegetables are excellent sources of carotenoids, nutrients that may play critical roles in the prevention of important human diseases such as cancer, cardiovascular disease [197, 198], and age-related macular degeneration [196]. Recent epidemiological and animal studies suggest a direct relationship between a high dietary intake of carotenoids from fruits and vegetables and a decreased risk of these disorders [199]. Studies have shown that carotenoids contribute to the yellow colour found in many fruits and vegetables. The conjugated double bonds and various functional groups of carotenoids affect the colour of fruits and vegetables. As a result, their colour ranges from yellow to red or orange in many fruits and vegetables [200]. Besides, the colour of the carotenoid can also be affected by whether the molecule is esterified or not.[201]. These pigments are isoprenoid compounds, biosynthesised by tail-to-tail linkage of two C_{20} geranyl diphosphate molecules. This produces the parent C_{40} carbon skeleton from which all the individual variations are derived [202]. Carotenoids are classified as (1) carotenes or carotenoid hydrocarbons, composed of only carbon and hydrogen, e.g., lycopene and *β*-carotene; and (2) xanthophylls or oxygenated carotenoids that may contain epoxy, carbonyl, hydroxy, methoxy or carboxylic acid functional groups (Figure 11). Examples of xanthophylls are violaxanthin (epoxy), canthaxanthin (oxo), zeaxanthin (hydroxy), spirilloxanthin (methoxy) and torularhodin (carboxylic acid) [203].

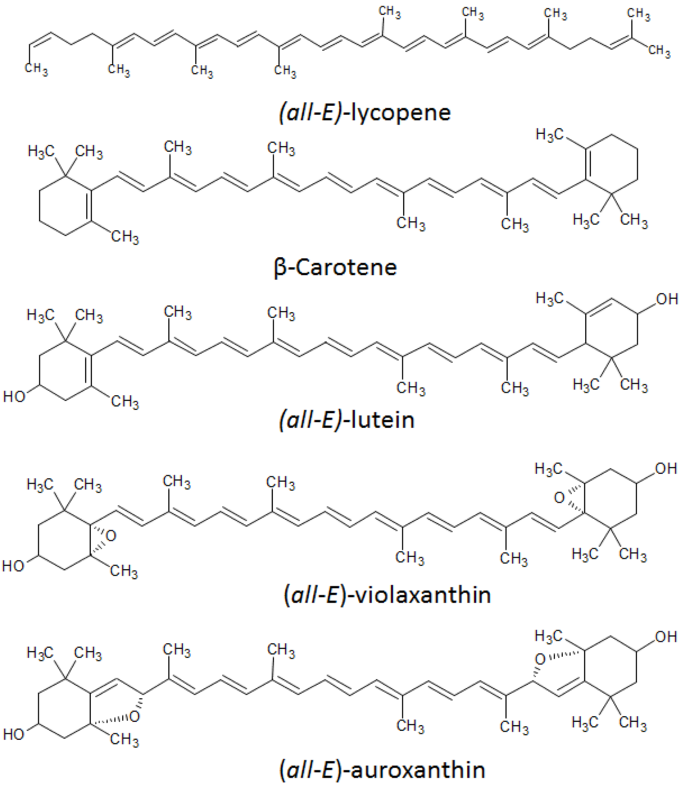


Figure 11. Scheme of an acyclic carotenoid (lycopene) and dicyclic carotenoid (β-carotene). Examples of different types of xantophylls. Hidroxycarotenoid ((*all-E*-)lutein) and epoxycarotenoids: 5,6-epoxide groups ((*all-E*) violaxanthin) and 5,8-furanoid groups ((*all-E*-)auroxanthin)

Cultivar variety, part of the plant consumed, stage of maturity, climate and geographic origin are factors that affect the carotenoid composition of fruits and vegetables, as well as postharvest handling, processing and storage [204]. In fruits and vegetables and their processed products as juices, great variety of carotenoids and their geometrical isomers have been described. The main carotenes found in fruits and vegetables are β-carotene, phytoene and lycopene highlighting carrots or apricots as sources of β-carotene or tomato as a fruit rich in lycopene. Among the hydroxycarotenoids lutein, zeaxanthin and β-cryptoxanthin are the most representative in fruits and vegetables, high contents of β-cryptoxanthin are described in chili, papaya and tangerine. High amounts of lutein and zeaxanthin have also been described in fruits such as oranges and peaches and vegetables such as pumpkins, spinach, kale and broccoli [109]. Remarkable quantities of epoxycarotenoids can also be found in fruits and vegetables, for example orange, peach and apple have a rich profile in violaxanthin, antheraxanthin and neoxanthin isomers. In this sense tropical fruits such as mango, camu and pequi have also high contents in epoxycarotenoids [205, 206]. Little attention has been paid to a detailed characterisation of by-products and fibres obtained from these food matrices. Carotenoids—usually their total content—have been addressed in by-products from carrot [207], orange dietary fibre [208] and by-products from tropical fruit [209].

Although carotenoids are fairly stable in natural food environments, they are much more labile when food cells are damaged as during pulping or cutting. Carotenoids are highly susceptible to external agents, such as heat, low pH, and light exposure, promoting changes in colour due to the rearrangement or formation of degradation compounds such as *cis*-isomers, epoxides, short chain products, and, in some cases, volatile compounds [210]. Contradictions in literature can be found in studies about the degradation of carotenoids during fruit and vegetable processing. In some publications, processing seems to increase the carotenoid content due to cell breakage. However, in other publications, a significant reduction of the carotenoid content occurs when a heat treatment is applied, with a high dependence of the processing time. The reason is attributed to oxidation of coloured carotenoids to non-coloured compounds [211-213]. The isomerisation *trans*-to-*cis* seems unequivocally a clear consequence of thermal processing. In red, yellow and orange fruits and vegetables, the major form is 13-*cis* followed by 9-*cis* and 15-*cis* isomers. In contrast, for processed green vegetables the main isomer is 9-*cis* followed by 13-*cis* [205, 210]. Another isomerisation reaction takes place when organic acids that are released during fruit juice processing, promote rearrangements of 5,6-epoxide groups to 5,8-furanoid groups of carotenoids [213, 214].

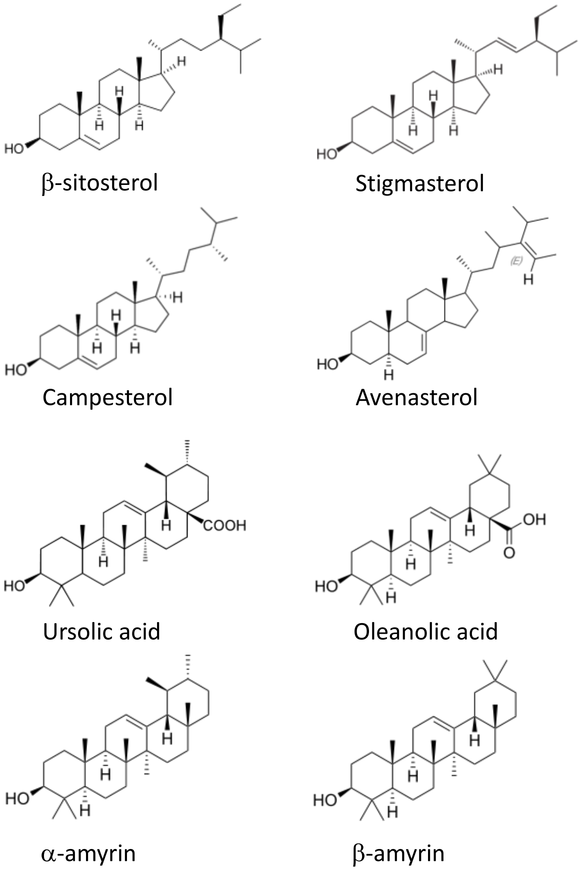
The extraction process of carotenoids is a critical stage in their analysis due to their high lability. Thus, degradation due to oxidative conditions, thermal decomposition or photodegradation, must be avoided. Consequently, extracting in lightless or dim light conditions, in the presence of antioxidants (such as dibutylhydroxytoluene (BHT)) and avoiding high temperatures is always recommended. Carotenoids are soluble in apolar solvents but because of the high moisture content of plant tissues, a preliminary extraction solvent miscible with water is generally necessary to allow the penetration of the solvent into the tissue [204]. Moreover, it has been found that the most efficient extraction solvents are composed by a slightly polar solvent, commonly methanol, ethanol or acetone, in addition to the nonpolar solvent such as ethyl acetate, hexane, diethyl ether, chloroform, etc. The extraction process usually includes a saponification step to hydrolyse the hydroxycarotenoids bonded as esters. This practice simplifies the resulting chromatogram [215].

Despite some spectroscopic techniques such as NMR [216] and NIR [217] have been used to identify these pigments, HPLC, using various detectors, has become the most common analytical method for determining carotenoid profiles, both qualitatively and quantitatively. NP and predominantly RP columns have been used in HPLC to separate carotenoids in fruits and vegetables. The C18 and C30 RP columns are the most commonly used for the analysis of carotenoids. The C30 phase [218-220] allows better separation of the less polar compounds, especially in resolution of carotenoid esters respect to C18 [112, 221]. Various solvent mixtures have been described as mobile phase, including water, methanol, acetone, isopropanol, ethyl acetate, chloroform, methyl-*tert*-butyl ether (MTBE), dichloromethane (DCM) and tetrahydrofuran (THF) depending on the stationary phase previously selected. UPLC is also applied to separate carotenoids, although C30 stationary phase is not already available to UPLC mode, so C18 are the columns used to separate the main carotenoids in fruits and vegetables when this technology is used. [129, 130, 222]. In addition, comprehensive LC has been applied to analyse some fruits and vegetables very rich in carotenoids. In these studies a NP column in the first dimension was combined with a C18 to carry out the rapid separation in second dimension [223, 224]. UV-Vis detectors are the most commonly used in the quantification and to obtain the spectrum used to identify the carotenoids. However, the similarity of spectra and chromatographic co-elutions has led to the need for other tools. Mass detectors have shown great advantages for the analysis of carotenoids, including elucidation of their structure on the basis of the molecular mass and their fragmentation pattern. The mainly used ionisation sources are ESI and APCI. APPI has also been studied with application of various dopants [225, 226]. APCI is the most successful ionization source used for xanthophylls and carotenes and it has also been applied in carotenoid esters analysis to elucidate the carotenoid and the fatty acid linked to it [224].

1.2.2.3. Triterpenoids and phytosterols.

Triterpenes are a class of chemical compounds composed by two chains of six isoprene units with $C_{30}H_{48}$ molecular formula. Triterpenoids are functionalised triterpenes and they commonly possess heteroatoms. Triterpenoids have been shown to possess numerous biological activities and display various pharmacological effects such as antiinflammatory, antiulcer, antibacterial, antiviral (including anti-HIV), hepatoprotective, immunomodulatory, hypolipidemic and cholesterol-lowering, antiatherosclerotic, wound-healing, anticoagulant and anticarcinogenic properties, combined with relatively low toxicity [227]. Sterols are a subgroup from steroids, which are derivatives of triterpenes with 26 or more carbon atoms that have undergone a characteristic type of rearrangement. Sterols of plants are called phytosterols and their chemical structure is similar to that of cholesterol. Plant sterols play an important role in the structure and function of plant cell membranes [228]. Plant sterols have long been known to have serum cholesterol lowering properties and also by their anticarcinogenic and anti-inflammatory properties [229, 230]. On the other hand, stanols are saturated sterols, having no double bonds in the sterol ring structure [231], with similar properties than phytosterols [232].

Total phytosterol content on a fresh weight are markedly lower in vegetables and fruits than in vegetable oils and cereals. However, their significance as dietary triterpenoid and phytosterol source has been shown to be considerable [233]. In fruits and vegetables, phytosterols and phytostanols in their free or esterified form, are compounds with low polarity that are found in abundance in parts as peels and seeds [234, 235]. Plant sterols and stanols have been described in fruits such as orange, tangerine and mango and in vegetables such as pea, cauliflower, broccoli and lettuce. The total phytosterol content in vegetables ranged 1-54 mg/100 g edible portion and phytosterol contents in fruits ranged 1-44 mg/100 g, the highest concentration being found in navel orange, tangerine, and mango [236-239]. The most common sterols in vegetables and fruit are 4-desmethylsterols such as sitosterol, campesterol, stigmasterol and avenasterols (Figure 12) [235]. Phytosterols may be oxidised during food processing and storage. Their degradation process has only been studied in vegetable oils and fats. In this type of samples, their degradation was shown to depend on the sterol structure, mainly unsaturation of the ring structure, temperature and matrix [233]. However, Soupas et al. tested various food treatments, included heat treatments, in different fats and oils and concluded that they don’t represent an important source of phytosterol oxides. [240]. Triterpenoids have also been described in several fruits and vegetables such as apple, berries, grape, tomato, grape, cabbage and eggplant [241, 242, 243]. The main triterpenoids in fruits and vegetables are ursolic acid, oleanolic acid and α-, β-, δ-amyrins (Figure 12) [227, 244]. Oleanolic and ursolic acids are commonly found in cuticular wax of fruits such as apple, persimmon, pear and olive [245]. In this way, apple peel and pomace and olive pomace have been described as sources, mainly in ursolic in and oleanolic acids, repectively [246, 247]. Considering the effect from processing, when tomato industrial byproducts from juice factory are compared with unprocessed tomatoes, it is found that the levels of triterpenoids are not diminished during processing [248].



β-sitosterol
Stigmasterol
Campesterol
Avenasterol
Ursolic acid
Oleanolic acid
α-amyrin
β-amyrin

Figure 12. Common sterols and triterpenoids in fruit and vegetables.

Phytosterols and triterpenoids in fruits and vegetables and their processed products can be isolated by solvent extraction with chloroform–methanol–water followed by alkaline or acid hydrolysis and chromatographic purification to obtain enriched total sterols. Capillary GC has been the technique of choice for the analysis of sterols and related compounds. Capillary columns offer shorter analysis times and lesser peak interference, improvement in component resolution, and high thermal stability compared with packed columns. Although the separation of sterols and stanols without derivatisation is possible, the resolution of a sterol and its corresponding stanol is not as good as that of their trimethylsilyl (TMS) or acetate derivatives. Thus, prior to capillary GC, sterols and stanols are usually transformed into derivatives that yield improved peak shape, resolution and sensitivity, and a higher stability for the thermally labile unsaturated sterols. The former are more suitable for the GC–MS characterisation and quantitation of sterols [227, 249-251]. HPLC coupled to MS has also been applied to determine sterols and triterpenoids. To optimize the separation of this lipophilic compounds, the mainly used columns were C18 eluting at high percentages of methanol or acetonitrile [220]. HPLC-MS using APCI source has become a successful technique to quantify and identify these compounds [244] because operating in product ion mode allows the unambiguous identification of unknown compounds including isomers elucidation [252].

1.3. References

[1] https://www.nutrition.org.uk/healthyliving/healthyeating/fruit-and-vegetables.html, (accesed on July 2016).

[2] J.C. Rickman, D.M. Barrett, C.M. Bruhn. Journal of the Science of Food and Agriculture, 87 (2007) 930.

[3] A. Oikawa, T. Otsuka, R. Nakabayashi, Y. Jikumaru, K. Isuzugawa, H. Murayama, K. Saito, K. Shiratake. PloS One, 10 (2015) 408.

[4] P. Nath, M. Bouzayen, A.K. Mattoo, J. claude Pech, Fruit ripening: physiology, signalling and genomics, CABI, 2014.

[5] G.B. Seymour, M. Poole, J.J. Giovannoni, G.A. Tucker, The molecular biology and biochemistry of fruit ripening, Wiley Online Library, 2013.

[6] A. Crozier, M.N. Clifford, H. Ashihara, Plant secondary metabolites: occurrence, structure and role in the human diet, John Wiley & Sons, 2008.

[7] M.E. Dauthy, Fruit and vegetable processing, Food and Agriculture Organization of the United Nations. Rome, 1995.

[8] IBISWorld, IBISWorld Industry Report, Global Fruit & Vegetables Processing, 2015.

[9] K.F. Kiple, The Cambridge world history of food. 2 (2000), Cambridge University Press, 2000.

[10] http://comtrade.un.org/pb/CommodityPagesNew.aspx?y=2014UN, Comtrade and UN Service Trade, (accesed on May 2016).

[11] http://www.ifc.org/wps/wcm/connect/b76bd080488552d8aca4fe6a6515-bb18/fruitandvg_PPAH.pdf?MOD=AJPERES, (accesed on July 2016).

[12] L. European Commission Council Directive 2001/112/EC of 20 December 2001 relating to fruit juices and certain similar products intended for human consumption. Off. J. Eur. Commun. 2002, L10,58-66.

[13] L. European Commission Council Directive 2001/112/EC of 19 April 2012 relating to fruit juices and certain similar products intended for human consumption. Off. J. Eur. Commun. 2012, L115, 1-11

[14] http://www.britishsoftdrinks.com/write/MediaUploads/Publications/BSDA - FRUIT JUICE GUIDANCE May 2016.pdf, (accesed on August 2016).

[15] http://www.zucasa.es/productos.aspx, (accesed on August 2016).

[16] http://www.indulleida.com/indexpeng.html, (accesed on August 2016).

[17] http://global.britannica.com/topic/fruit-processing, (accesed on June 2016).

[18] G.M. Sapers, J.R. Gorny, A.E. Yousef, Microbiology of fruits and vegetables, CRC Press, 2005.

[19] I. Körmendy, Fruit Processing, in: Handbook of Fruits and Fruit Processing, Blackwell Publishing, 2007.

[20] http://www.cobell.co.uk/index.php?page=56, (accesed on August 2016).

[21] R.P. Bates, J. Morris, P.G. Crandall, Principles and practices of small-and medium-scale fruit juice processing, Food & Agriculture Org., 2001.

[22] M. Colin-Henrion, G. Cuvelier, C. Renard, Stewart Postharvest Review, 3 (2007) 1.

[23] P.J. Fellows, Food processing technology: principles and practice, Elsevier, 2009.

[24] J.E. Lozano, Fruit manufacturing, Springer, 2006.

[25] D. Heatherbell, Confructa, 28 (1984) 192.

[26] J.E. Lozano, Scientific Basis, Engineering Properties, and Deteriorative Reactions of Technological Importance in Fruit Manufacturing:, Springer, 2006.

[27] E. Hernandez, Food Engineering, 3 (2009) 69.

[28] M.S. Rahman, Handbook of food preservation, CRC press, 2007.

[29] L. Kitinoja, A.A. Kader, Small-scale postharvest handling practices: a manual for horticultural crops, University of California, Davis, Postharvest Technology Research and Information Center, 2002.

[30] D.M. Barrett, L. Somogyi, H.S. Ramaswamy, Processing fruits: science and technology, CRC Press, 2004.

[31] http://ecoursesonline.iasri.res.in/mod/page/view.php?id=123997, (accesed on August 2016).

[32] B. Grout, G. Morris, M. McLellan, Freezing of fruit and vegetables, in: Food Freezing, Springer, 1991

[33] E.D. Lowe, D.R. Buckmaster, Biocycle: Journal of Composting and Recycling, 1 (1995) 78.

[34] G. Laufenberg, B. Kunz, M. Nystroem, Bioresource Technology, 87 (2003) 167.

[35] A. Nawirska, M. Kwaśniewska, Food Chemistry, 91 (2005) 221.

[36] N. O'Shea, E.K. Arendt, E. Gallagher, Innovative Food Science & Emerging Technologies, 16 (2012) 1.

[37] A.P. do Espírito Santo, N.S. Cartolano, T.F. Silva, F.A. Soares, L.A. Gioielli, P. Perego, A. Converti, M.N. Oliveira, International Journal of Food Microbiology, 154 (2012) 135.

[38] T. de Moraes Crizel, A. Jablonski, A. de Oliveira Rios, R. Rech, S.H. Flôres, LWT-Food Science and Technology, 53 (2013) 9.

[39] N. Balasundram, K. Sundram, S. Samman, Food Chemistry, 99 (2006) 191.

[40] I. Marañón, R. Virto, J. García, A. Martínez, M. Cubero, P. Echevarría, J. Larrauri, Conference paper: Targeted carotenoid metabolite profiling in transgenic cereals and correlations with carotenoid gene expression, (2012).

[41] N. Hounsome, B. Hounsome, D. Tomos, G. Edwards-Jones, Journal of food Science, 73 (2008) 48.

[42] P.E. Shaw, Handbook of sugar separations in foods by HPLC, CRC Press, 1988.

[43] I.H.-D. Belitz, I.W. Grosch, Amino acids, peptides, proteins, in: Food Chemistry, Springer, 1999.

[44] A.A. Kader, Journal of the Science of Food and Agriculture, 88 (2008) 1863.

[45] V. Gökmen, N. Artık, J. Acar, N. Kahraman, E. Poyrazoğlu, European Food Research and Technology, 213 (2001) 194.

[46] F. Vaillant, M. Cisse, M. Chaverri, A. Perez, M. Dornier, F. Viquez, C. Dhuique-Mayer, Innovative Food Science & Emerging Technologies, 6 (2005) 213.

[47] T. Garde-Cerdán, M. Arias-Gil, A.R. Marsellés-Fontanet, C. Ancín-Azpilicueta, O. Martín-Belloso, Food Control, 18 (2007) 473.

[48] F. Chinnici, U. Spinabelli, C. Riponi, A. Amati, Journal of Food Composition and Analysis, 18 (2005) 121.

[49] A. Schieber, Introduction to Food, in: Modern techniques for food authentication, Elsevier, 2008.

[50] F. Zeng, J. Ou, Y. Huang, Q. Li, G. Xu, Z. Liu, S. Yang, Food Analytical Methods, 8 (2015) 428.

[51] P. Hernández-Orte, M. Ibarz, J. Cacho, V. Ferreira, Chromatographia, 58 (2003) 29.

[52] W. Liming, Z. Jinhui, X. Xiaofeng, L. Yi, Z. Jing, Journal of Food Composition and Analysis, 22 (2009) 242.

[53] S. Guo, J.-a. Duan, D. Qian, Y. Tang, Y. Qian, D. Wu, S. Su, E. Shang, Journal of Agricultural and Food Chemistry, 61 (2013) 2709.

[54] S. Özcan, H.Z. Şenyuva, Journal of Chromatography A, 1135 (2006) 179.

[55] I. Molnár-Perl, Journal of Chromatography A, 845 (1999) 181.

[56] I. Molnár-Perl, Journal of Chromatography A, 891 (2000) 1.

[57] S. Tisza, I. Molnár-Perl, M. Friedman, P. Sass, Journal of High Resolution Chromatography, 19 (1996) 54.

[58] S. Tisza, P. Sass, I. Molnár-Perl, Journal of Chromatography A, 676 (1994) 461.

[59] I. Molnár-Perl, A. Vasanits, K. Horváth, Chromatographia, 48 (1998) 111.

[60] M. Cerdán-Calero, J.M. Sendra, E. Sentandreu, Journal of Chromatography A, 1241 (2012) 84.

[61] H. Kelebek, S. Selli, Journal of the Science of Food and Agriculture, 91 (2011) 1855.

[62] B.M. Silva, P.B. Andrade, G.C. Mendes, R.M. Seabra, M.A. Ferreira, Journal of Agricultural and Food Chemistry, 50 (2002) 2313.

[63] J. Van Duynhoven, A. Voda, M. Witek, H. Van As, Annual Reports on NMR Spectroscopy, 69 (2010) 145.

[64] M. Guđjónsdóttir, P.S. Belton, Magnetic resonance in food science: Challenges in a changing world, Royal Society of Chemistry, 2009.

[65] F. Bertocchi, M. Paci, Journal of Agricultural and Food Chemistry, 56 (2008) 9317.

[66] M. Valentini, M. Ritota, C. Cafiero, S. Cozzolino, L. Leita, P. Sequi, Magnetic Resonance in Chemistry, 49 (2011) S121.

[67] S. Braun, H. Kalinowski, S. Berger, L. Ernst, K. Ibrom, Angewandte Chemie-German Edition, 111 (1999) 1227.

[68] N. Marigheto, L. Venturi, B. Hills, Postharvest Biology and Technology, 48 (2008) 331.

[69] P. Belton, I. Delgadillo, A. Gil, P. Roma, F. Casuscelli, I. Colquhoun, M. Dennis, M. Spraul, Magnetic Resonance in Chemistry, 35 (1997) S52.

[70] P.S. Belton, I.J. Colquhoun, E.K. Kemsley, I. Delgadillo, P. Roma, M.J. Dennis, M. Sharman, E. Holmes, J.K. Nicholson, M. Spraul, Food Chemistry, 61 (1998) 207.

[71] D. Capitani, A.P. Sobolev, A. Tomassini, F. Sciubba, F.R. De Salvador, L. Mannina, M. Delfini, Journal of Agricultural and Food Chemistry, 61 (2012) 1718.

[72] N. Hernández-Sánchez, B. Hills, P. Barreiro, N. Marigheto, Postharvest Biology and Technology, 44 (2007) 260.

[73] P.S. Belton, I. Delgadillo, E. Holmes, A. Nicholls, J.K. Nicholson, M. Spraul, Journal of Agricultural and Food Chemistry, 44 (1996) 1483.

[74] J.T. Vogels, L. Terwel, A.C. Tas, F. van den Berg, F. Dukel, J. van der Greef, Journal of Agricultural and Food Chemistry, 44 (1996) 175.

[75] G.E. Pereira, J.-P. Gaudillere, C. van Leeuwen, G. Hilbert, M. Maucourt, C. Deborde, A. Moing, D. Rolin, Analytica Chimica Acta, 563 (2006) 346.

[76] I.F. Duarte, B.J. Goodfellow, A.M. Gil, I. Delgadillo, Spectroscopy Letters, 38 (2005) 319.

[77] A.M. Gil, I.F. Duarte, I. Delgadillo, I.J. Colquhoun, F. Casuscelli, E. Humpfer, M. Spraul, Journal of Agricultural and Food Chemistry, 48 (2000) 1524.

[78] M. Koda, K. Furihata, F. Wei, T. Miyakawa, M. Tanokura, Journal of Agricultural and Food Chemistry, 60 (2012) 1158.

[79] D. Capitani, L. Mannina, N. Proietti, A. Sobolev, A. Tomassini, A. Miccheli, M. Di Cocco, G. Capuani, R. De Salvador, M. Delfini, Talanta, 82 (2010) 1826.

[80] C. Deborde, M. Maucourt, P. Baldet, S. Bernillon, B. Biais, G. Talon, C. Ferrand, D. Jacob, H. Ferry-Dumazet, A. de Daruvar, Metabolomics, 5 (2009) 183.

[81] F. Mounet, M. Lemaire-Chamley, M. Maucourt, C. Cabasson, J.-L. Giraudel, C. Deborde, R. Lessire, P. Gallusci, A. Bertrand, M. Gaudillère, Metabolomics, 3 (2007) 273.

[82] A.P. Sobolev, G. Testone, F. Santoro, C. Nicolodi, M.A. Iannelli, M.E. Amato, A. Ianniello, E. Brosio, D. Giannino, L. Mannina, Journal of Agricultural and Food Chemistry, 58 (2010) 6928.

[83] A. Tardieu, W. De Man, H. This, Analytical and Bioanalytical Chemistry, 398 (2010) 3139.

[84] M. Ritota, F. Marini, P. Sequi, M. Valentini, Journal of Agricultural and Food Chemistry, 58 (2010) 9675.

[85] A. Cazor, C. Deborde, A. Moing, D. Rolin, H. This, Journal of Agricultural and Food Chemistry, 54 (2006) 4681.

[86] G. Del Campo, I. Berregi, R. Caracena, J.I. Santos, Analytica Chimica Acta, 556 (2006) 462.

[87] A. Caligiani, D. Acquotti, G. Palla, V. Bocchi, Analytica Chimica Acta, 585 (2007) 110.

[88] L.I. Nord, P. Vaag, J.Ø. Duus, Analytical Chemistry, 76 (2004) 4790.

[89] Y.B. Monakhova, B. Schütz, H. Schäfer, M. Spraul, T. Kuballa, H. Hahn, D.W. Lachenmeier, Accreditation and Quality Assurance, 19 (2014) 17.

[90] M. Spraul, B. Schütz, P. Rinke, S. Koswig, E. Humpfer, H. Schäfer, M. Mörtter, F. Fang, U.C. Marx, A. Minoja, Nutrients, 1 (2009) 148.

[91] C.K. Chow, Fatty acids in foods and their health implications, CRC Press, 2007.

[92] M.W. Pariza, J. Yager, S. Goldfarb, J.A. Gurr, S. Yanagi, S.H. Grossman, J.E. Becker, T.A. Barber, V.R. Potter, Biochemical, autoradiographic, and electron microscopic studies of adult rat liver parenchymal cells in primary culture, in: Gene expression and carcinogenesis in cultured liver, Academic Press New York, 1975.

[93] H. Hansen, International news on fats, oils and related materials, (1990).

[94] J. Dziezak, Food Technology, 40 (1986) 94.

[95] R.R. Stewart, J.D. Bewley, Plant Physiology, 65 (1980) 245.

[96] J. Wu, H. Gao, L. Zhao, X. Liao, F. Chen, Z. Wang, X. Hu, Food Chemistry, 103 (2007) 88.

[97] K. Golden, O. Williams, Journal of Chromatographic Science, 39 (2001) 243.

[98] E. Ryan, K. Galvin, T. O'Connor, A. Maguire, N. O'Brien, International Journal of Food Sciences and Nutrition, 57 (2006) 219.

[99] B. Fuchs, R. Süß, K. Teuber, M. Eibisch, J. Schiller, Journal of Chromatography A, 1218 (2011) 2754.

[100] A. Tomàs, M. Tor, G. Villorbina, R. Canela, M. Balcells, J. Eras, Journal of Chromatography A, 1216 (2009) 3290.

[101] J. Eras, J. Ferran, B. Perpiña, R. Canela, Journal of Chromatography A, 1047 (2004) 157.

[102] USDA Human Nutrition Information Service, Agriculture Handbook No. 8, Composition of Foods, (1984).

[103] E. Arena, S. Campisi, B. Fallico, E. Maccarone, Journal of Agricultural and Food Chemistry, 46 (1998) 4138.

[104] P. May, Chlorophyll, University of Bristol. School of Chemistry, 2014.

[105] M. Ferruzzi, V. Böhm, P. Courtney, S. Schwartz, Journal of food science, 67 (2002) 2589.

[106] M.G. Ferruzzi, J. Blakeslee, Nutrition Research, 27 (2007) 1.

[107] S.J. Schwartz, T.V. Lorenzo, Critical Reviews in Food Science & Nutrition, 29 (1990) 1.

[108] A. Humphrey, Journal of food science, 69 (2004) 422.

[109] J. Burns, P.D. Fraser, P.M. Bramley, Phytochemistry, 62 (2003) 939.

[110] F. Khachik, G.R. Beecher, N.F. Whittaker, Journal of Agricultural and Food Chemistry, 34 (1986) 603.

[111] Y. Suzuki, Y. Shioi, Journal of Agricultural and Food Chemistry, 51 (2003) 5307.

[112] M. Zapata, F. Rodriguez, J.L. Garrido, Marine Ecology Progress Series, 195 (2000) 29.

[113] F. Canjura, S. Schwartz, R. Nunes, Journal of Food Science, 56 (1991) 1639.

[114] S.J. Schwartz, S.L. Woo, J.H. Von Elbe, Journal of Agricultural and Food Chemistry, 29 (1981) 533.

[115] M.I. Minguez-Mosquera, B. Gandul-Rojas, J. Minguez-Mosquera, Journal of Agricultural and Food Chemistry, 42 (1994) 1089.

[116] European Parliament and Council Directive 94/36/EC of 30 June 1994 on colours for use in foodstuffs, Official Journal of the Commission of the European Communities,, 237 (1994) 13.

[117] European Food Safety Authority (EFSA), Scientific Opinion on reevaluation of copper complexes of chlorophylls (E141(i)) and chlorophyllins (E141(ii)) as food additives, 13 (2015) 4151.

[118] K. Östbring, M. Rayner, I. Sjöholm, J. Otterström, P.-Å. Albertsson, S.C. Emek, C. Erlanson-Albertsson, Food & function, 5 (2014) 2157.

[119] X. Hu, A. Tanaka, R. Tanaka, Plant methods, 9 (2013) 1.

[120] M.J. Scotter, L. Castle, D. Roberts, Food Additives and Contaminants, 22 (2005) 1163.

[121] S. Wright, S. Jeffrey, R. Mantoura, Evaluation of methods and solvents for pigment extraction in Phytoplankton pigments in oceanography: guidelines to modern methods. Monographs on Oceanographic Methodology, UNESCO, 1997.

[122] A.R.-B. de Quirós, H.S. Costa, Journal of Food Composition and Analysis, 19 (2006) 97.

[123] J. Folch, M. Lees, G. Sloane-Stanley, Journal of Biological Chemistry, 226 (1957) 497.

[124] L. Almela, J.A. Fernández-López, M.a.J. Roca, Journal of Chromatography A, 870 (2000) 483.

[125] S. Huang, C. Hung, W. Wu, B. Chen, Journal of Pharmaceutical and Biomedical Analysis, 48 (2008) 105.

[126] T.H. Kao, C.J. Chen, B.H. Chen, Talanta, 86 (2011) 349.

[127] N.L. Puspitasari-Nienaber, M.G. Ferruzzi, S.J. Schwartz, Journal of the American Oil Chemists' Society, 79 (2002) 633.

[128] K.K. Chebrolu, G.G. Yousef, R. Park, Y. Tanimura, A.F. Brown, Journal of Chromatography B, 1001 (2015) 41.

[129] B. Chauveau-Duriot, M. Doreau, P. Nozière, B. Graulet, Analytical and bioanalytical chemistry, 397 (2010) 777.

[130] W. Fu, M. Magnúsdóttir, S. Brynjólfson, B.Ø. Palsson, G. Paglia, Analytical and bioanalytical chemistry, 404 (2012) 3145.

[131] R. Mantoura, C. Llewellyn, Analytica chimica acta, 151 (1983) 297.

[132] M. Roca, L. Gallardo-Guerrero, M.a.I. Mínguez-Mosquera, B. Gandul Rojas, Journal of Agricultural and Food Chemistry, 58 (2009) 51.

[133] Y.J. Park, W.S. Kim, S.H. Ko, D.S. Lim, H.J. Lee, W.Y. Lee, D.W. Lee, Journal of liquid chromatography & related technologies, 26 (2003) 3183.

[134] R. Aparicio-Ruiz, K.M. Riedl, S.J. Schwartz, Journal of Agricultural and Food Chemistry, 59 (2011) 11100.

[135] M.M. Mendes-Pinto, A.C. Silva Ferreira, C. Caris-Veyrat, P. Guedes de Pinho, Journal of Agricultural and Food Chemistry, 53 (2005) 10034.

[136] S.M. Milenković, J.B. Zvezdanović, T.D. Anđelković, D.Z. Marković, Advanced technologies, 1 (2012) 16.

[137] D. Hornero-Méndez, B. Gandul-Rojas, M.I. Mínguez-Mosquera, Food research international, 38 (2005) 1067.

[138] M. Roca, B. Gandul-Rojas, L. Gallardo-Guerrero, M.I. Mínguez-Mosquera, Journal of the American Oil Chemists' Society, 80 (2003) 1237.

[139] M. Oberhuber, J. Berghold, W. Mühlecker, S. Hörtensteiner, B. Kräutler, Helvetica Chimica Acta, 84 (2001) 2615.

[140] T. Ngo, Y. Zhao, Journal of food science, 72 (2007) C397.

[141] J.-L. Lafeuille, S.p. Lefèvre, J. Lebuhotel, Journal of Agricultural and Food Chemistry, 62 (2014) 1926.

[142] G. Goldberg, Plants: diet and health, John Wiley & Sons, 2008.

[143] I. Ignat, I. Volf, V.I. Popa, Food Chemistry, 126 (2011) 1821.

[144] V. Popa, M. Dumitru, I. Volf, N. Anghel, Industrial Crops and Products, 27 (2008) 144.

[145] F.A. Tomás-Barberán, M.N. Clifford, Journal of the Science of Food and Agriculture, 80 (2000) 1073.

[146] B. Lapornik, M. Prošek, A.G. Wondra, Journal of Food Engineering, 71 (2005) 214.

[147] S. Arranz, F. Saura-Calixto, S. Shaha, P.A. Kroon, Journal of Agricultural and Food Chemistry, 57 (2009) 7298.

[148] N. Raper, B. Perloff, L. Ingwersen, L. Steinfeldt, J. Anand, Journal of Food Composition and Analysis, 17 (2004) 545.

[149] J. Gry, L. Black, F.D. Eriksen, K. Pilegaard, J. Plumb, M. Rhodes, D. Sheehan, M. Kiely, P.A. Kroon, Trends in Food Science & Technology, 18 (2007) 434.

[150] V. Neveu, J. Perez-Jimenez, F. Vos, V. Crespy, L. Du Chaffaut, L. Mennen, C. Knox, R. Eisner, J. Cruz, D. Wishart, Database, 2010 (2010) 24.

[151] J. Pérez-Jiménez, V. Neveu, F. Vos, A. Scalbert, Journal of Agricultural and Food Chemistry, 58 (2010) 4959.

[152] R. Zadernowski, S. Czaplicki, M. Naczk, Food Chemistry, 112 (2009) 685.

[153] L. Bravo, Nutrition Reviews, 56 (1998) 317.

[154] F. Shahidi, M. Naczk, Phenolics in food and nutraceuticals, CRC press, 2003.

[155] L. Porter, Condensed tannins, in: Natural products of woody plants, Springer, 1989

[156] A.E. Hagerman, Vanillin Method. Department of Chem. and Biochem. Miami University, 2002.

[157] D. Delmas, A. Lançon, D. Colin, B. Jannin, N. Latruffe, Current drug targets, 7 (2006) 423.

[158] N. Alper, K.S. Bahçeci, J. Acar, Journal of Food Processing and Preservation, 29 (2005) 357.

[159] T. Hernandez, N. Auśn, B. Bartolome, L. Bengoechea, I. Estrella, C. Gómez-Cordovés, Zeitschrift für Lebensmitteluntersuchung und-Forschung A, 204 (1997) 151.

[160] A. Gil-Izquierdo, M.I. Gil, F. Ferreres, Journal of Agricultural and Food Chemistry, 50 (2002) 5107.

[161] Y. Klopotek, K. Otto, V. Böhm, Journal of Agricultural and Food Chemistry, 53 (2005) 5640.

[162] Y.-J. Hong, D.M. Barrett, A.E. Mitchell, Journal of Agricultural and Food Chemistry, 52 (2004) 2366.

[163] I. Klimczak, M. Małecka, M. Szlachta, A. Gliszczyńska-Świgło, Journal of Food Composition and Analysis, 20 (2007) 313.

[164] T.M. Rababah, M.A. Al-Mahasneh, I. Kilani, W. Yang, M.N. Alhamad, K. Ereifej, M. Aloudatt, Journal of the Science of Food and Agriculture, 91 (2011) 1096.

[165] W. Peschel, F. Sánchez-Rabaneda, W. Diekmann, A. Plescher, I. Gartzía, D. Jiménez, R. Lamuela-Raventos, S. Buxaderas, C. Codina, Food Chemistry, 97 (2006) 137.

[166] H.H. Wijngaard, C. Rößle, N. Brunton, Food Chemistry, 116 (2009) 202.

[167] M. Coll, L. Coll, J. Laencina, F. Tomas-Barberan, Zeitschrift für Lebensmitteluntersuchung und-Forschung A, 206 (1998) 404.

[168] M. Naczk, F. Shahidi, Journal of Chromatography A, 1054 (2004) 95.

[169] P. Ramírez, T. Fornari, F.J. Señoráns, E. Ibanez, G. Reglero, The Journal of Supercritical Fluids, 35 (2005) 128.

[170] R. Alonso-Salces, E. Korta, A. Barranco, L. Berrueta, B. Gallo, F. Vicente, Journal of Chromatography A, 933 (2001) 37.

[171] T. Inoue, S. Tsubaki, K. Ogawa, K. Onishi, J.-i. Azuma, Food Chemistry, 123 (2010) 542.

[172] M. Herrera, M.L. De Castro, Journal of Chromatography A, 1100 (2005) 1.

[173] M. Nardini, E. Cirillo, F. Natella, D. Mencarelli, A. Comisso, C. Scaccini, Food Chemistry, 79 (2002) 119.

[174] P. Mattila, J. Kumpulainen, Journal of Agricultural and Food Chemistry, 50 (2002) 3660.

[175] Y. Velioglu, G. Mazza, L. Gao, B. Oomah, Journal of Agricultural and Food Chemistry, 46 (1998) 4113.

[176] J.C. Sánchez-Rangel, J. Benavides, J.B. Heredia, L. Cisneros-Zevallos, D.A. Jacobo-Velázquez, Analytical Methods, 5 (2013) 5990.

[177] M. Naczk, F. Shahidi, Journal of Pharmaceutical and Biomedical Analysis, 41 (2006) 1523.

[178] J. Lee, C. Rennaker, R.E. Wrolstad, Food Chemistry, 110 (2008) 782.

[179] L. Gu, M. Kelm, J.F. Hammerstone, G. Beecher, D. Cunningham, S. Vannozzi, R.L. Prior, Journal of Agricultural and Food Chemistry, 50 (2002) 4852.

[180] O. Gürbüz, D. Göçmen, F. Dagˇdelen, M. Gürsoy, S. Aydin, İ. Şahin, L. Büyükuysal, M. Usta, Food Chemistry, 100 (2007) 518.

[181] R. Capasso, Phytochemical Analysis, 10 (1999) 299.

[182] B. Abad-García, S. Garmón-Lobato, L. Berrueta, B. Gallo, F. Vicente, Journal of Mass Spectrometry, 44 (2009) 1017.

[183] B. Abad García, S. Garmón Lobato, L.A. Berrueta, B. Gallo, F. Vicente, Rapid Communications in Mass Spectrometry, 23 (2009) 2785.

[184] A. Schieber, W. Ullrich, R. Carle, Innovative Food Science & Emerging Technologies, 1 (2000) 161.

[185] D.J. Zeeb, B.C. Nelson, K. Albert, J.J. Dalluge, Analytical chemistry, 72 (2000) 5020.

[186] J.B. Jean-Denis, R. Pezet, R. Tabacchi, Journal of Chromatography A, 1112 (2006) 263.

[187] J. Wang, P. Sporns, Journal of Agricultural and Food Chemistry, 47 (1999) 2009.

[188] Y.-Z. Cai, J. Xing, M. Sun, Z.-Q. Zhan, H. Corke, Journal of Agricultural and Food Chemistry, 53 (2005) 9940.

[189] I.M. Abu-Reidah, D. Arráez-Román, R. Quirantes-Piné, S. Fernández-Arroyo, A. Segura-Carretero, A. Fernández-Gutiérrez, Food Research International, 46 (2012) 108.

[190] D. Fracassetti, C. Costa, L. Moulay, F.A. Tomás-Barberán, Food Chemistry, 139 (2013) 578.

[191] R. Quirantes-Piné, L. Funes, V. Micol, A. Segura-Carretero, A. Fernández-Gutiérrez, Journal of Chromatography A, 1216 (2009) 5391.

[192] C.E. da Silva, P. Vandenabeele, H.G. Edwards, L.F.C. de Oliveira, Analytical and Bioanalytical Chemistry, 392 (2008) 1489.

[193] R. Ferrer-Gallego, J.M. Hernández-Hierro, J.C. Rivas-Gonzalo, M.T. Escribano-Bailón, LWT-Food Science and Technology, 44 (2011) 847.

[194] I. Berregi, J.I. Santos, G. del Campo, J.I. Miranda, J.M. Aizpurua, Analytica Chimica Acta, 486 (2003) 269.

[195] N.-E. Es-Safi, S. Guyot, P.-H. Ducrot, Journal of Agricultural and Food Chemistry, 54 (2006) 6969.

[196] D.M. Snodderly, The American Journal of Clinical Nutrition, 62 (1995) 1448S.

[197] R. Peto, R. Doll, J.D. Buckley, M. Sporn, Nature, 290 (1981) 201.

[198] V.A. Kirsh, S.T. Mayne, U. Peters, N. Chatterjee, M.F. Leitzmann, L.B. Dixon, D.A. Urban, E.D. Crawford, R.B. Hayes, Cancer Epidemiology Biomarkers & Prevention, 15 (2006) 92.

[199] B. Pool-Zobel, A. Bub, H. Müller, I. Wollowski, G. Rechkemmer, Carcinogenesis, 18 (1997) 1847.

[200] H.-E. Khoo, K.N. Prasad, K.-W. Kong, Y. Jiang, A. Ismail, Molecules, 16 (2011) 1710.

[201] D.E. Breithaupt, A. Bamedi, Journal of Agricultural and Food Chemistry, 49 (2001) 2064.

[202] R. Vélez, S. Maiam, Thesis: Targeted carotenoid metabolite profiling in transgenic cereals and correlations with carotenoid gene expression, 2012.

[203] S. Rivera, R. Canela-Garayoa, Journal of Chromatography A, 1224 (2012) 1.

[204] D.B. Rodriguez-Amaya, A guide to carotenoid analysis in foods, ILSI press Washington, DC, 2001.

[205] A.Z. Mercadante, D.B. Rodriguez-Amaya, Journal of Agricultural and Food Chemistry, 46 (1998) 128.

[206] V.V. de Rosso, A.Z. Mercadante, Journal of Agricultural and Food Chemistry, 55 (2007) 5062.

[207] B.H. Chen, Y.C. Tang, Journal of Agricultural and Food Chemistry, 46 (1998) 2312.

[208] T. de Moraes Crizel, A. Jablonski, A. de Oliveira Rios, R. Rech, S.H. Flôres, LWT - Food Science and Technology, 53 (2013) 9.

[209] L.M.R.d. Silva, E.A.T.d. Figueiredo, N.M.P.S. Ricardo, I.G.P. Vieira, R.W.d. Figueiredo, I.M. Brasil, C.L. Gomes, Food Chemistry, 143 (2014) 398.

[210] A.Z. Mercadante, Food colorants: Chemical and functional properties, (2007) 213.

[211] C.M. Stinco, R. Fernández-Vázquez, M.L. Escudero-Gilete, F.J. Heredia, A.J. Meléndez-Martínez, I.M. Vicario, Journal of Agricultural and Food Chemistry, 60 (2012) 1447.

[212] J.G. Provesi, C.O. Dias, E.R. Amante, Food Chemistry, 128 (2011) 195.

[213] L.Q. Zepka, A.Z. Mercadante, Food Chemistry, 117 (2009) 28.

[214] A.J. Meléndez-Martínez, I.M. Vicario, F.J. Heredia, Journal of Food Composition and Analysis, 20 (2007) 638.

[215] K. Amorim-Carrilho, A. Cepeda, C. Fente, P. Regal, TrAC Trends in Analytical Chemistry, 56 (2014) 49.

[216] S. Tiziani, S.J. Schwartz, Y. Vodovotz, Journal of Agricultural and Food Chemistry, 54 (2006) 6094.

[217] H. Schulz, M. Baranska, R. Baranski, Biopolymers, 77 (2005) 212.

[218] P. Dugo, M. Herrero, D. Giuffrida, C. Ragonese, G. Dugo, L. Mondello, Journal of Separation Science, 31 (2008) 2151.

[219] P. Dugo, V. Škeríková, T. Kumm, A. Trozzi, P. Jandera, L. Mondello, Analytical Chemistry, 78 (2006) 7743.

[220] D. Giuffrida, P. Dugo, G. Dugo, G. Torre, L. Mondello, Natural product communications, 6 (2011) 1817.

[221] A.A. Franke, C.M. Morrison, L.J. Custer, X. Li, J.F. Lai, Journal of Chromatography A, 1301 (2013) 1.

[222] B.P. Arathi, P.R.R. Sowmya, K. Vijay, P. Dilshad, B. Saikat, V. Gopal, R. Lakshminarayana, Food Analytical Methods, 8 (2015) 1.

[223] F. Cacciola, P. Donato, D. Giuffrida, G. Torre, P. Dugo, L. Mondello, Journal of Chromatography A, 1255 (2012) 244.

[224] P. Dugo, M. Herrero, T. Kumm, D. Giuffrida, G. Dugo, L. Mondello, Journal of Chromatography A, 1189 (2008) 196.

[225] S.M. Rivera, P. Christou, R. Canela-Garayoa, Mass Spectrometry Reviews, 33 (2014) 353.

[226] S. Rivera, F. Vilaró, R. Canela, Analytical and bioanalytical chemistry, 400 (2011) 1339.

[227] A. Szakiel, C. Pączkowski, F. Pensec, C. Bertsch, Phytochemistry Reviews, 11 (2012) 263.

[228] A.H. Lichtenstein, R.J. Deckelbaum, Circulation, 103 (2001) 1177.

[229] W. Ling, P. Jones, Life Sciences, 57 (1995) 195.

[230] T.P. Carr, M. Ash, A. Brown, Nutrition and Dietary Supplements, 2 (2010) 59.

[231] R.R. Watson, V.R. Preedy, Bioactive Food as Dietary Interventions for Cardiovascular Disease: Bioactive Foods in Chronic Disease States, Academic Press, 2012.

[232] F. O'Neill, A. Brynes, R. Mandeno, N. Rendell, G. Taylor, M. Seed, G. Thompson, Nutrition, Metabolism and Cardiovascular Diseases, 14 (2004) 133.

[233] P.C. Dutta, Phytosterols as functional food components and nutraceuticals, CRC Press, 2003.

[234] J.L. Harwood, 6 Plant Lipid Metabolism, in: Plant biochemistry, Academic Press, 1997

[235] V. Piironen, D.G. Lindsay, T.A. Miettinen, J. Toivo, A.M. Lampi, Journal of the Science of Food and Agriculture, 80 (2000) 939.

[236] T. Bacchetti, S. Masciangelo, V. Bicchiega, E. Bertoli, G. Ferretti, Mediterranean Journal of Nutrition and Metabolism, 4 (2011) 165.

[237] W.S. Jeong, P. Lachance, Journal of Food Science, 66 (2001) 278.

[238] L. Normen, M. Johnsson, H. Andersson, Y. Van Gameren, P. Dutta, European Journal of Nutrition, 38 (1999) 84.

[239] H. Jun-Hua, Y. Yue-Xin, F. Mei-Yuan, Biomedical and Environmental Sciences, 21 (2008) 449.

[240] L. Soupas, L. Huikko, A.-M. Lampi, V. Piironen, European Food Research and Technology, 222 (2006) 266.

[241] T. Cui, J.-Z. Li, H. Kayahara, L. Ma, L.-X. Wu, K. Nakamura, Journal of Agricultural and Food Chemistry, 54 (2006) 4574.

[242] N. Han, M. Bakovic, Journal of Bioanalysis & Biomedicine, 12 (2015) 1.

[243] I. Mezine, H. Zhang, C. Macku, R. Lijana, Journal of Agricultural and Food Chemistry, 51 (2003) 5639.

[244] K. Naumoska, I. Vovk, Journal of Chromatography A, 1381 (2015) 229.

[245] Y. Lv, I.I. Tahir, M.E. Olsson, Journal of the Science of Food and Agriculture, 96 (2015) 2161.

[246] M. Perez-Camino, A. Cert, Journal of Agricultural and Food Chemistry, 47 (1999) 1558.

[247] R. Shalini, D. Gupta, Journal of Food Science and Technology, 47 (2010) 365.

[248] N. Kalogeropoulos, A. Chiou, V. Pyriochou, A. Peristeraki, V.T. Karathanos, LWT-Food Science and Technology, 49 (2012) 213.

[249] M. Lagarda, G. Garcia-Llatas, R. Farré, Journal of Pharmaceutical and Biomedical Analysis, 41 (2006) 1486.

[250] Ł.P. Haliński, M. Paszkiewicz, M. Gołębiowski, P. Stepnowski, Journal of food composition and analysis, 25 (2012) 74.

[251] S. Bauer, E. Schulte, H.-P. Thier, European Food Research and Technology, 220 (2005) 5.

[252] M. Martelanc, I. Vovk, B. Simonovska, Journal of Chromatography A, 1216 (2009) 6662.

CHAPTER 2. OBJECTIVES

The general objective of this Doctoral Thesis is to improve the existing chromatographic methodologies to analyse metabolite compounds in fruit derivatives from the agroindustry.

To achieve the general objective, three secondary objectives have been considered:

The first one is the development of rapid and reliable analytical methods to quantify and identify carotenoids and chlorophylls in processed fruit and vegetable products. To achieve this objective, new approaches in chromatographic techniques will be assessed.

The second objective is the application of analytical methods for the characterisation of processed fruit and vegetable products metabolome and tracing its degradation compounds. In this line, the characterisation of processed fibres from the juice industry has been considered.

The third objective is the application and improvement of analytical methods to authenticate commercial monovarietal fruit juices.

CHAPTER 3. EXPERIMENTAL, RESULTS AND DISCUSSION

3.1. Development of UPLC-PDA-MS/MS methods to determine carotenoids, chlorophylls and their derivatives in processed fruit and vegetable products

This chapter is focused on the development of UPLC-PDA-MS/MS methods for the determination of carotenoids, chlorophylls and its derivatives in fruit and vegetable products. With the use of the developed methods, the chromatographic resolution, identification and quantification of complex mixtures containing up to 27 carotenoid and 48 chlorophyll derivatives was achieved.

The development of an analytical method to identify and quantify carotenoids in both freshly homemade and processed juice is necessary because these compounds are important for the complete description of their nutrient profiles along the industrial process. The lability of carotenoids requires the development of a rapid and sensitive analytical method and most of the previously published ones require long analysis time and/or have poor separation capacity [1, 2]. Few studies have reported UPLC methodologies and none of them has achieved the complex separation of 5,6- and 5,8-epoxycarotenoids and their isomers together with the separation of hydroxycarotenoids and carotenes [3, 4]. In this section, an improved method for epoxycarotenoids, which are the least retained compounds, is also included. This method is based on the greater peak efficiency obtained by UPLC and has allowed to obtain better chromatographic resolution between these compounds.

The analytical method described in this chapter has been applied to study the differences in the carotenoid profile of commercial and freshly made fruit products (peach purees and pear juices). According to the literature 5,6-epoxycarotenoids are the main carotenoids to define the profile of freshly made samples while they do not usually appear in commercial samples [5]. Then, this study pays special attention to the rearrangements of 5,6-epoxycarotenoids into 5,8-epoxycarotenoids during the corresponding industrial processes. Simultaneously, the behaviour of other carotenoids is monitored to detect any selective degradation during processing. In addition, this study includes the optimisation of an extraction method for carotenoids using freeze-dried samples to reduce time and solvent consumption. Commonly, reported methods are based on a solid-liquid extraction followed by solvent partitioning [6]. In this study, the extraction efficiencies have been optimised in our matrices using various extraction solvents and mixtures including methanol, methanol:THF, ethanol, ethanol:hexane and acetone. On the other hand, the capacity of this method to differentiate among monovarietal peach and pear juices was tested in either commercial or freshly made ones.

Chlorophyll and their derivatives are also used as markers in food processing, so that it is necessary to have methods to identify and quantify chlorophylls and their derivatives in processed foodstuffs. Similar to the study conducted for carotenoids, a rapid method for determining chlorophyll and their derivatives was also developed. This method has been applied to processed foods, allowing tracking chlorophyll degradation.

So far, previously published methodologies require long analysis time and sometimes a low number of chlorophyll compounds are detected. Few references can be found in the literature where UPLC methodology is used to separate chlorophyll derivatives, but they are applied only to the separation of phytylated compounds [7, 8]. In this study, we sought to develop an UPLC method that allows simultaneous analysis of chlorophylls and chlorophyll derivatives, namely phytilated and dephytilated, metallized and demetallized and Cu2+ complexes of chlorophylls including food colouring known as E-141(i) and E-141(ii). Previously reported chromatographic separations have displayed a number of drawbacks such as low retention and poor resolution between some compounds. We have solved these separation problems using a reversed-phase UPLC with on column ion pairing reagent. In previous publications, results using APCI ionisation are described for the most non-polar compounds. Moreover, other works have highlighted ionisation problems in both APCI and ESI [9, 10], so we decided to test both sources of atmospheric pressure ionisation.

We also tested the developed methodology on different types of samples, which cover the main types of chlorophyll and their derivatives. Among them, we included teas, in which chlorophylls and derivatives have been described as a marker of processing and storage quality [11]. The method was also tested in jams and juices from "green" vegetables and fruits. These samples are characterised by higher processing using various temperature stages such as blanching, canning and retorting, which led to a high degradation of chlorophyll and their derivatives. In addition, some samples are additivated with colorants or Cu^{2+} for a regreening effect, which added difficulty to the analysis.

The results of these studies have been published in the *Journal of Chromatography A 1331 (2014) 90-99,* and submitted to *Journal of Chromatography A*.

References.

[1] A. Meléndez-Martínez, G. Britton, I. Vicario, F. Heredia, Food Research International, 38 (2005) 931.

[2] C. Cortés, M.J. Esteve, A. Frígola, F. Torregrosa, Journal of Agricultural and Food Chemistry, 52 (2004) 2203.

[3] A.J. Meléndez-Martínez, I.M. Vicario, F.J. Heredia, Journal of Food Composition and Analysis, 20 (2007) 638.

[4] B. Chauveau-Duriot, M. Doreau, P. Nozière, B. Graulet, Analytical and Bioanalytical Chemistry, 397 (2010) 777.

[5] A. Asai, M. Terasaki, A. Nagao, The Journal of Nutrition, 134 (2004) 2237.

[6] S. Rivera, R. Canela-Garayoa, Journal of Chromatography A, 1224 (2012) 1.

[7] W. Fu, M. Magnúsdóttir, S. Brynjólfson, B.Ø. Palsson, G. Paglia, Analytical and Bioanalytical Chemistry, 404 (2012) 3145.

[8] I. Guzman, G.G. Yousef, A.F. Brown, Journal of Agricultural and Food Chemistry, 60 (2012) 7238.

[9] R.L. Airs, B.J. Keely, Rapid Communications in Mass Spectrometry, 14 (2000) 125.

[10] S.M. Milenković, J.B. Zvezdanović, T.D. Anđelković, D.Z. Marković, Advanced technologies, 1 (2012) 16.

[11] Y. Suzuki, Y. Shioi, Journal of Agricultural and Food Chemistry, 51 (2003) 5307.

3.1.1. Ultra performance liquid chromatography analysis to study the changes in the carotenoid profile of commercial monovarietal fruit juices

ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS TO STUDY THE CHANGES IN THE CAROTENOID PROFILE OF COMMERCIAL MONOVARIETAL FRUIT JUICES

Antoni Delpino-Rius [a], Jordi Eras [a,b,*], Alexis Marsol-Vall [a], Francisca Vilaró [b], Mercè Balcells [a,*,] Ramon Canela-Garayoa [a]

[a] Chemistry Department, ETSEA, University of Lleida, 25198-Lleida, Spain

[b] Scientific Technical Services, University of Lleida, 25198-Lleida, Spain

Journal of Chromatography A 1331 (2014) 90-99

Keywords: Carotenoid profile; UPLC; Monovarietal fruit juice; Epoxycarotenoid; Hydroxycarotenoid.

ABSTRACT

We have developed an analytical method that allows the simultaneous determination of epoxycarotenoids, hydroxycarotenoids and carotenes in monovarietal fresh homemade and industrially processed fruit products. Analyses were carried out using ultra performance liquid chromatography (UPLC). The extraction method was optimized using methanol as the first extraction solvent for lyophilized samples followed by a saponification step. Recoveries ranged between 75% and 104% depending on the compound. Repeatability was better than 10% for all compounds (%RSD, n = 3). The chromatographic analysis takes less than 17 min. In this short period, up to 27 carotenoids were identified in apple, peach and pear products. The developed method allowed us to differentiate juice from six varieties of apple by their carotenoid profile. Moreover, the methodology allows us to differentiate the carotenoid profiles from commercial juices and homemade fresh peach and pear juices, as well as to study the rearrangements of 5,6- to 5,8-epoxycarotenoids.

1. Introduction.

The quality of fruit juices is determined by several parameters; among these are colour, stability and the presence of nutrients with health benefits. Carotenoids are phytochemicals with anti-oxidant properties, and they can reduce the risk of lifestyle-related diseases, including cancer. In the human diet, these compounds are present in many foodstuffs of both animal and plant origin, but mainly in fruit and vegetables [1, 2]. Moreover, the carotenoid profile could be used for authentication purposes, to differentiate not only fruits, but also their varieties. With the aim of offering new products to customers, the fruit juice industry has recently developed monovarietal juices, and interest in the carotenoid profile has increased for the assessment of juice quality. By comparing these profiles before and after industrial juice production, changes could be used as indicators of the care taken in the process. Various authors have reported that heat treatments cause undesirable biochemical changes and loss of bioactive compounds in freshly made juice [3, 4]. Carotenoids are considered labile compounds; their higher electron density, caused by the extended double-bond conjugation present, makes them more prone to degradation. They are also susceptible to rearrangements. In this regard, the rearrangement of the 5,6-epoxide to 5,8-epoxide in acidic conditions has been described in fruit juices [5, 6]. In this context, rapid analytical methods are needed to identify and quantify carotenoids in both freshly homemade juice and processed varieties. The carotenoid profile would allow assessment of the loss of nutritional value of a monovarietal juice after processing.

Several approaches have been developed to extract carotenoids, such as the pressurized solvent [7-9], supercritical carbon dioxide [10, 11] or Soxhlet [12] techniques. However, the most widely used method to analyse these compounds in fruit juices and purees is solid–liquid extraction [13, 14]. Solvent extraction is a time-consuming step that requires large volumes of organic solvents. This effect could be minimized by freeze-drying the samples. Carotenoids differ greatly in solubility; consequently, the choice of extraction solvent and the use of a saponification step will have strong effects on carotenoid recoveries. Various extraction solvents have been used for this purpose, depending on the sample and carotenoids addressed [15, 16].

To date, reversed-phase high-performance liquid chromatography (HPLC), mainly with C30 stationary phases[17-19], but also C18 stationary phases [20, 21] and normal phase combined with reversed-phase [22, 23] have been the main tool used for the analysis of carotenoids in fruit products. Recently, ultra performance liquid chromatography (UPLC) technology has also been used to analyse carotenoids in various matrices. As the UPLC C30 stationary phase columns are not commercially available, C18 columns have been used [24-27]. Commercial standards, when available, are applied to achieve identification. Therefore, UV-vis fine structure, mass spectra and published information on retention times in distinct chromatographic systems are also useful for this purpose. Although MS cannot distinguish stereoisomers, this technique is considered reliable for the assignment of carotenoid structures.

Here, we sought to develop a rapid and reliable analytical method to determine the carotenoid profile of apple, pear and peach monovarietal juices and purees. With this in mind, a UPLC method was developed to obtain a fast separation for the simultaneous determination of major carotenoids in freshly made and commercial juices and purees. Extraction of carotenoids was performed using freeze-dried samples to reduce time and solvent consumption. In addition, we used the different operation modes of a tandem mass spectrometer to confirm the molecular mass and the characteristic fragmentation patterns of the carotenoids tentatively identified.

2. Material and methods.

2.1. Reagents, solvents and carotenoid standards.

Ethanol, 2-propanol, methanol (MeOH), ethyl acetate, hexane, diethyl ether, tetrahydrofuran (THF), acetonitrile (ACN) and acetone (HPLC grade purity) were supplied by J.T.Baker (Deventer, The Netherlands). The chromatographic solvents were ACN, methanol (LC–MS grade), also from J.T.Baker, and water was purified in a Milli-Q system from Millipore (Bedford, MA, USA). Sodium chloride was provided by Fluka (Buchs SG, Switzerland), potassium hydroxide by Panreac (Barcelona, Spain) and ascorbic acid by Acros (Pittsburgh, PA, USA). THF and diethyl ether were stored under argon atmosphere at 4 ºC to prevent formation of peroxides.

β-Carotene, (all-*E*)-lutein and β-cryptoxanthin were purchased from Sigma-Aldrich Fine Chemicals (Madrid, Spain). (all-*E*)-Zeaxanthin was supplied by Fluka. Phytoene, (all-*E*)-violaxanthin, (9′*Z*)-neoxanthin and (all-*E*)-antheraxanthin were purchased from Carote Nature (Lupsingen, Switzerland).

All stock standard solutions were prepared in ACN:MeOH:acetone (47:20:33, v/v/v) and stored at –80 ºC. Carotenoid concentrations were determined spectrophotometrically using a UV2 UV-vis spectrometer (ATI Unicam, Cambridge, UK). The values of $A_{1\%\ 1\ \mathrm{cm}}$, described in the bibliography, were used to quantify each carotenoid stock solution [28]. Working solutions were prepared from stock solutions by sampling an aliquot and diluting it with the injection solvent (ACN/MeOH/acetone (47:20:33, v/v/v)).

2.2. Samples.

Approximately 1 kg of randomly chosen apples (*Malus domestica*) of the cultivars Fuji, Golden Delicious, Granny Smith, Pink Lady, Red Delicious and Royal Gala varieties were purchased from a local market. Apples were cored and four pieces were squeezed with a household juicer (JU200; Moulinex, Lyon, France).

Six commercial peach purees (*Prunus persica*) of Spring Lady and Miraflores cultivars and their corresponding fresh fruit were provided by a local juice company (Zucasa, Fraga, Spain). The same company provided six pear juices of Conference and Blanquilla cultivars and their corresponding fresh fruit. Pears were cored and peaches were stoned. Four pieces of pear were squeezed with a household juicer and four pieces of peach were homogenized in a blender (Grindomix GM 200; Retsch, Haan, Germany) at 5000 rpm for 2 min.

In the squeezer or blender steps, ascorbic acid (ca. 10 g/kg) was added to prevent oxidation. It is known that ascorbic acid can promote rearrangements in some carotenoids in aqueous media, consequently the samples were processed immediately, as is discussed below [5].

Aliquots between 1.5 and 5 g, depending on the sample, of apple and pear juices and peach puree were weighed in a vessel, immediately frozen at –80 ºC and lyophilized at -50ºC, 1.1 P for 24 h in a Cryodos-50 lyophilizer (Telstar, Terrassa, Spain). Finally, lyophilized samples were powdered in the same vessel, and stored at –20 ºC until analysis.

2.3. Extraction procedure.

Powdered juices and purees were continuously shaken at 5000 rpm (Multi Reax; Heidolph, Schwabach, Germany) for 10 min in a dark room in the same vessel used for lyophilization with 4 mL of one of the following solvents: *Solvent 1*, methanol

[19, 29]; *Solvent 2*, methanol:THF 1:1 [13, 20, 30]; *Solvent 3*, ethanol [31]; *Solvent 4*, ethanol:hexane 4:3 [15, 32]; and *Solvent 5*, acetone [19]. For all solvents, except solvent 4, an equal volume of hexane (4 mL) was added after the first extraction, and the vial was shaken again as before. Next, 2.3 mL of a solution of NaCl (10% w/v) was added. The mixture was shaken for 15 min and cooled at 4 ºC. The mixture was centrifuged at 2700 × *g* for 3 min at 4 ºC, and the organic layer was recovered. The aqueous phase was re-extracted with 2 mL of hexane:diethyl ether (3:1, v/v) for 10 min and centrifuged in the same conditions. The organic solutions were combined in a 5-mL glass tube and dried under nitrogen stream at room temperature.

The dry residue was saponified in a shaking incubator (211DS; Labnet, Edison, NJ, USA) for 60 min at 55 ºC under N_2 using 1 mL of a solution of 6% KOH in methanol (w/v). After the addition of 1 mL of sodium chloride solution (10%, w/v), the mixture was put in a freezer for 15 min. Then, 2.5 mL of hexane:diethyl ether (3:1, v/v) was added, and the mixture was vortexed and centrifuged at 433 × *g* for 3 min (this step was repeated until the aqueous phase was colourless). The organic layers were combined and the solvent was removed under nitrogen stream. The residue was stored at –80 ºC under nitrogen until UPLC analysis. The dry residue was dissolved in the injection solvent [33] immediately prior to analysis.

2.4. Recovery assays.

Carotenoids were quantified by the external standard calibration method using a PDA detector. Recoveries were assessed in pear spiking samples with available standards. Three samples were fortified before extraction and another three after extraction. To evaluate the effect of the saponification step on the recoveries of (9′*Z*)-neoxanthin and (all-*E*)-violaxanthin, samples were spiked just prior at the saponification step following the same process mentioned above.

2.5. Ultra performance liquid chromatography.

Chromatographic analysis was performed on a Waters ACQUITY UPLC™ system (Waters, Milford, MA, USA) consisting of ACQUITY UPLC™ binary solvent manager and ACQUITY UPLC™ sample manager, coupled to a photodiode array (PDA) 2996 detector. Compounds were separated with an ACQUITY UPLC™ BEH C18 column (1.7 μm; 2.1 mm × 100 mm) (Waters, Manchester, UK) and a gradient system with the mobile phase consisting of solvent

A, ACN–MeOH: (7:3, v/v) and solvent B, H_2O 100%. The linear gradient was as follows: 0–0.6 min, 25% B, 0.5 mL/min (isocratic); 0.6–6.5 min, 4.9% B 0.5 mL/min (linear gradient); 6.5–7.5 min, 0% B, 0.7 mL/min (linear gradient); 7.5–13.6 min, 0% B, 0.7 mL/min (isocratic); 13.6–14.1 min, 25% B, 0.5 mL/min (linear gradient); and 14.1–16.6 min, 25% B, 0.5 mL/min (isocratic). Weak and strong needle solvents were ACN–MeOH (7:3, v/v) and 2-propanol, respectively. The injection volume was 5 µL and the column was kept at 32 ºC while the temperature in the sample manager was maintained at 25 ºC. Wavelengths used in the quantification of each carotenoid by the PDA detector are included in the Supplementary Data. Instrument control, data acquisition and processing were carried out using MassLynx™ software (version 4.1; Waters, USA).

2.6. Mass spectrometry.

MS analysis was carried out on a Waters ACQUITY TQD tandem quadrupole mass spectrometer (Waters, UK). The instrument was operated using an atmospheric pressure chemical ionization (APCI) source in positive ion mode. The APCI parameters were as follows: corona voltage 4.0 kV; extractor voltage 3 V; source temperature 150 ºC; probe temperature 450 ºC; desolvation temperature 150 ºC; cone gas (nitrogen) flow 10 L/h; and desolvation gas (also nitrogen) flow 150 L/h. Collision-induced dissociation was achieved using argon as the collision gas at a flow rate of 0.15 mL/min in the collision cell. Data acquisition was performed using MassLynx 4.1 software (Waters, USA).

3. Results and discussion.

3.1. Extraction optimization.

To achieve a simple and reliable method to extract the carotenoids, the process was previously optimized using peach puree. Because the water content of fruit and juice samples is between 80% and 90%, the samples were lyophilized to reduce the volume of extraction solvents required. Despite lyophilization could provoke a partial degradation of some carotenoids, mostly lycopene [34], lyophilization is recognized by some authors as a suitable technique for preserving carotenoids [35, 36], and generally does not excessively decompose these compounds [31, 37-41]. We have lyophilized taking all possible preventive actions to minimize degradation considering also, the absence of lycopene in our samples.

In order to assess extraction efficiencies on the lyophilized materials, three samples of ca. 200 mg of freeze-dried material (approx. 1 g of peach puree) were extracted with the five tested solvents. The process was continued as described in the Material and methods section. Only the saponification step was skipped. Each final organic solution containing the corresponding carotenoids was dried under nitrogen stream and reconstituted with hexane. Three replicates were used for each solvent. The total carotenoid contents, expressed as β-carotene, were determined spectrophotometrically using $A_{1\%\ 1\ \mathrm{cm}}$ in hexane at 450 nm [28]. The percentage of non-extracted carotenoids was calculated by assigning a value of 100% to the method that led to the highest total carotenoid content.

The MeOH/THF mixture and methanol extractions achieved the highest extraction of the major carotenoids in the samples, followed by ethanol, which extracted 12% less (Table 1). The ethanol/hexane mixture and 100% acetone extractions showed the poorest performance, with extraction yields around 30% lower than the most efficient extraction solvents. In fact, only the MeOH/THF mixture and methanol allowed complete decolouration of the peach samples (see picture in Supplementary Data). Given that THF exhibits greater toxicity and a tendency, as an ether compound, to form peroxides, which are known to catalyse carotenoid decomposition, methanol was chosen as the starting extraction solvent in subsequent assays.

Table 1. Total carotenoids extracted with the five starting solvents.

Number	Starting solvent	Total carotenoids (µg/g fresh matter) [a,b]	% Non-extracted carotenoids
1	Methanol	12.43 ± 0.21	1
2	Methanol:THF 1:1	12.54 ± 0.24	0
3	Ethanol	11.04 ± 0.24	12
4	Ethanol:hexane 4:3	8.41 ± 0.10	33
5	Acetone	8.53 ± 0.36	32

[a] Mean and SD corresponding to *n* = 3.

[b] Expressed as β-carotene.

3.2. UPLC optimization.

Here, we tested two columns, namely the ACQUITY UPLC BEH C18 and the ACQUITY UPLC HSS T3, both C18 reversed-phase, with 100 mm × 2.1 mm dimensions. To evaluate column performance, the gradient described by Rivera et al. [33] was applied to samples and standards. The HSS T3 column retains a greater amount of polar carotenoids and β-carotene than the BEH C18 column [24, 25]. The retention time of β-carotene in HSS T3 was delayed by more than 8 min in comparison with that of BEH C18. This delay resulted in a dramatic increase in the analysis time and deterioration in the limit of detection (LOD) and limit of quantification (LOQ) because of the peak broadening. Thus, the ACQUITY BEH C18 column was chosen to perform the carotenoid analyses in fruit juices and purees.

The gradient was optimized on water concentration (25%) and slope of the first gradient to attain optimum separation without causing excessive peak broadening. The column temperature was set at 32 ºC, keeping it below the 35 ºC maximum recommended for the analysis of carotenoids to prevent their degradation[42]. In previous studies, low-polar solvents, such as dichloromethane [24], ethyl acetate [27], chloroform [26] and *tert*-butyl methyl ether [25], had been used to decrease retention time. Nevertheless, Waters (USA) recommends not using these solvents in their UPLC systems [43]. Fu et al. achieved the separation of the main algae carotenoids in 20 min but using tert-butyl methyl ether and a column temperature of 45ºC [25]. With our optimized method, the separation of the main carotenoids was shortened to 16.5 min, being the fastest separation until now. In addition, the method allowed separation of the hydroxycarotenoids (all-*E*)-zeaxanthin and (all-*E*)-lutein (R_s = 1.45), which differ only in the position of one double bond. The approach reported by Fu et al. [25] required MS detection to quantify (all-*E*)-lutein and (all-*E*)-zeaxanthin; however, MS detection is not the most suitable option to quantify carotenoids.

Up to now, UPLC has only been used to determine the commercially available hydroxycarotenoids ((all-*E*)-zeaxanthin, (all-*E*)-lutein, β-cryptoxanthin) and β-carotene in nectarine and pumpkin [27]. In the present study, we focused on the complex separation of 5,6- and 5,8-epoxycarotenoids and their isomers, besides the hydroxycarotenoids and carotenes. The final gradient allowed the complete separation of the standards available (Figure 1). Moreover, a suitable separation of the main geometrical isomers of violaxanthin and neoxanthin present in fruit and 5,8-epoxycarotenoids, such as luteoxanthin, neochrome, mutatoxanthin and auroxanthin, was achieved. This study is the first to describe the separation of epoxycarotenoids present in fruit or fruit products by UPLC and greatly improves the previously reported HPLC methods [5,21]. This improvement is due to greater peak efficiency obtained by UPLC for epoxycarotenoids, which are the least retained compounds, and allowed us to obtain better chromatographic resolution between the epoxycarotenoids.

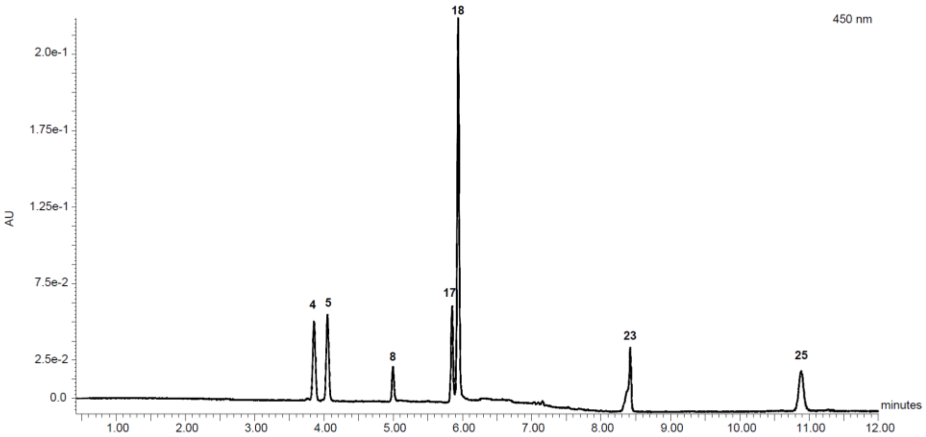


Figure 1. Chromatographic analysis of a mixture of carotenoid standards ((9'Z)-neoxanthin (4), (all-E)-violaxanthin (5), (all-E)-antheraxanthin (8), (all-E)-zeaxanthin (17), (all-E)-lutein (18), β-cryptoxanthin (23), β-carotene (25), numbered according to Table 3. Detection measured at 450 nm.

3.3. Method validation and recoveries.

The method was validated by determining the instrumental LOQ and LOD, repeatability (expressed as relative standard deviation (RSD)) and recoveries (%) using spiked samples with available standards. Table 2 shows the LODs, recoveries and method repeatability. LODs ranged from 0.022 μg/mL to 0.16 μg/mL depending on the compound, and were determined considering the three-fold signal/noise ratio approach. LOQs ranged from 0.07 μg/mL to 0.52 μg/mL. These limits are comparable with or slightly better than those reported previously [25].

Recoveries were evaluated in pear because this sample had the less complex carotenoid profile. Despite the temperature of 55 ºC in the saponification step, the recoveries ranged from 89% to 104% for hydroxycarotenoids, (all-*E*)-antheraxanthin and β-carotene. These recoveries are similar to those reported in the literature [13]. Diepoxycarotenoids (9′*Z*)-neoxanthin and (all-*E*)-violaxanthin presented slightly lower recoveries of 75% and 82%, respectively. To explain these lower recoveries, it should be considered that these two compounds are more polar than the epoxycarotenoid (all-*E*)-antheraxanthin. This higher polarity of these free carotenoids may hamper their recovery from the spiked sample using the methanol extraction followed by hexane and hexane/diethyl ether partition processes. Nevertheless, it must be considered that these epoxycarotenoids are mainly esterified in fruit juices [44, 45]. Consequently, the recovery of (9′*Z*)-neoxanthin and (all-*E*)-violaxanthin as esters could be higher than that found in this spiking study due to their lower polarity. To evaluate recoveries when carotenoids are released, both free diepoxycarotenoids ((9′*Z*)-neoxanthin and (all-*E*)-violaxanthin) were spiked at the saponification step and the recoveries exceeded 90% (Table 2).

Table 2. LODs, recoveries and RSDs (%, *n* = 3) for carotenoid standards.

Carotenoid	LOD (μg/mL)	LOQ (μg/mL)	Recoveries (%, *n* = 3)	Repeatability (%RSD, *n* = 3)
(9′*Z*)-Neoxanthin	0.031	0.10	82 (93) [a]	7 (6) [a]
(all-*E*)-Violaxanthin	0.031	0.10	75 (98) [a]	4 (7) [a]
(all-*E*)-Antheraxanthin	0.029	0.10	93	4
(all-*E*)-Zeaxanthin	0.022	0.07	96	4
(all-*E*)-Lutein	0.029	0.10	93	4
β-Cryptoxanthin	0.060	0.20	93	4
β-Carotene	0.157	0.52	104	5

[a] In brackets, recoveries and repeatability associated with the saponification step.

3.4. Identification.

Identification of many carotenoids with the corresponding standard was limited because these standards are not commercially available. We therefore used various tools to identify the maximum number of carotenoids. The published data about chromatographic behaviour on C18 [20, 21] and C30 [17-19] phases together with the UV-vis absorption spectra (namely, the position of the absorption maxima and the spectral fine structure) were used to identify the carotenoids. Also published data in relation to the main carotenoids in the studied matrices, was used for identification purposes (Table 3). Although some carotenoids have a very similar chemical composition and structure, MS confirmed carotenoid identification by providing the molecular weight and fragmentation patterns using APCI in positive ionization [46].

Table 3. Chromatography, UV-vis and MS parameters of carotenoids found in the samples.

Peak	**Carotenoid**	t_r **(min)**	λ_{max} **(nm)**	**%(III/II)** [a]
1	(all-*E*)-Neoxanthin [c]	3.24	418;442;471	90
2	Neochrome A [c,d]	3.33	400;423;451	95
3	Neochrome B [c,d]	3.70	399;423;451	95
4	(9′*Z*)-Neoxanthin [b]	3.79	329;414;438;466	89
5	(all-*E*)-Violaxanthin [b]	4.00	417;442;471	93
6	(all-*E*)-Luteoxanthin A [c,d]	4.55	401;424;450	98
7	(all-*E*)-Luteoxanthin B [c,d]	4.75	401;424;450	98
8	(all-*E*)-Antheraxanthin [b]	4.89	422;448;475	62
9	Auroxanthin A [c,d]	4.99	384;403;428	100
10	Auroxanthin B [c,d]	5.07	384;403;428	100
11	Mutatoxanthin A [c,d]	5.41	408;430;456	60
12	(9*Z*)-Luteoxanthin A [c,d]	5.46	313;396;418;445	98
13	Auroxanthin C [c,d]	5.58	384;403;428	100
14	(9*Z*)-Violaxanthin [c]	5.55	328;414;437;466	92
15	(9*Z*)-Luteoxanthin B [c,d]	5.61	313;397;419;444	98
16	Mutatoxanthin B [c,d]	5.74	408;430;456	60
17	(all-*E*)-Zeaxanthin [b]	5.76	428;453;478	19
18	(all-*E*)-Lutein [b]	5.85	424;447;475	61
19	Lutein isomer [c]	6.96	332;419;449;471	
20	Lutein isomer [c]	7.02	332;419;449;468	
21	Lutein isomer [c]	7.08	332;419;449;469	36
22	(all-*E*)-α-Cryptoxanthin [c]	8.03	420;449;476	59
23	(all-*E*)-β-Cryptoxanthin [b]	8.37	424;452;479	18
24	ζ-Carotene [c]	10.35	381;402;426	94
25	β-Carotene [b]	10.86	422;452;479	11
26	Phytofluene [c]	11.00	332;349;369	84
27	Phytoene [b]	11.66	275;287;299	

[a] Fine structure; the relationship between the peak heights of the longest wavelength absorption band (III) and the middle absorption band (II), measured from the minimum between the two peaks, and expressed as a percentage.

Table 3. Cont.

Peak	**Carotenoid**	**Precursor ion [M+H]$^+$ (*m/z*)**	**Product ion 1 ^{d} (*m/z*)**	**Product ion 2 ^{e} (*m/z*)**
1	(all-*E*)-Neoxanthin [c]	601	167 (20)	583 (10)
2	Neochrome A [c,d]			
3	Neochrome B [c,d]			
4	(9′*Z*)-Neoxanthin [b]	601	167 (20)	583 (10)
5	(all-*E*)-Violaxanthin [b]	601	93 (45)	133 (40)
6	(all-*E*)-Luteoxanthin A [c,d]	601	93 (45)	133 (40)
7	(all-*E*)-Luteoxanthin B [c,d]	601	93 (45)	133 (40)
8	(all-*E*)-Antheraxanthin [b]	585	93 (55)	105 (45)
9	Auroxanthin A [c,d]	601	93 (45)	133 (40)
10	Auroxanthin B [c,d]	601	93 (45)	133 (40)
11	Mutatoxanthin A [c,d]	585	93 (55)	105 (45)
12	(9*Z*)-Luteoxanthin A [c,d]	601	93 (45)	133 (40)
13	Auroxanthin C [c,d]	601	93 (45)	133 (40)
14	(9*Z*)-Violaxanthin [c]	601	93 (45)	133 (40)
15	(9*Z*)-Luteoxanthin B [c,d]	601	93 (45)	133 (40)
16	Mutatoxanthin B [c,d]	585	93 (55)	105 (45)
17	(all-*E*)-Zeaxanthin [b]	568	551 (10)	135 (30)
18	(all-*E*)-Lutein [b]	568	551 (10)	135 (30)
19	Lutein isomer [c]			
20	Lutein isomer [c]			
21	Lutein isomer [c]			
22	(all-*E*)-α-Cryptoxanthin [c]			
23	(all-*E*)-β-Cryptoxanthin [b]	553	461 (15)	119(35)
24	ζ-Carotene [c]			
25	β-Carotene [b]	537	445 (15)	119(40)
26	Phytofluene [c]	543	93 (45)	69 (45)
27	Phytoene [b]	545	81 (35)	69 (35)

[b] Identification considering the UV-vis spectrum and specific mass fragments of the standard compounds.

[c] Tentative identification based on UV-vis spectrum, MS, fine structure and HPLC/UHPLC published data.

[d] Because it has not been possible to assign the chiral configuration of the 8 or 8′ carbon in these peaks, through UV-vis absorption spectra and MS, they have been named as A, B and C.

[e] In brackets collision energy (kV).

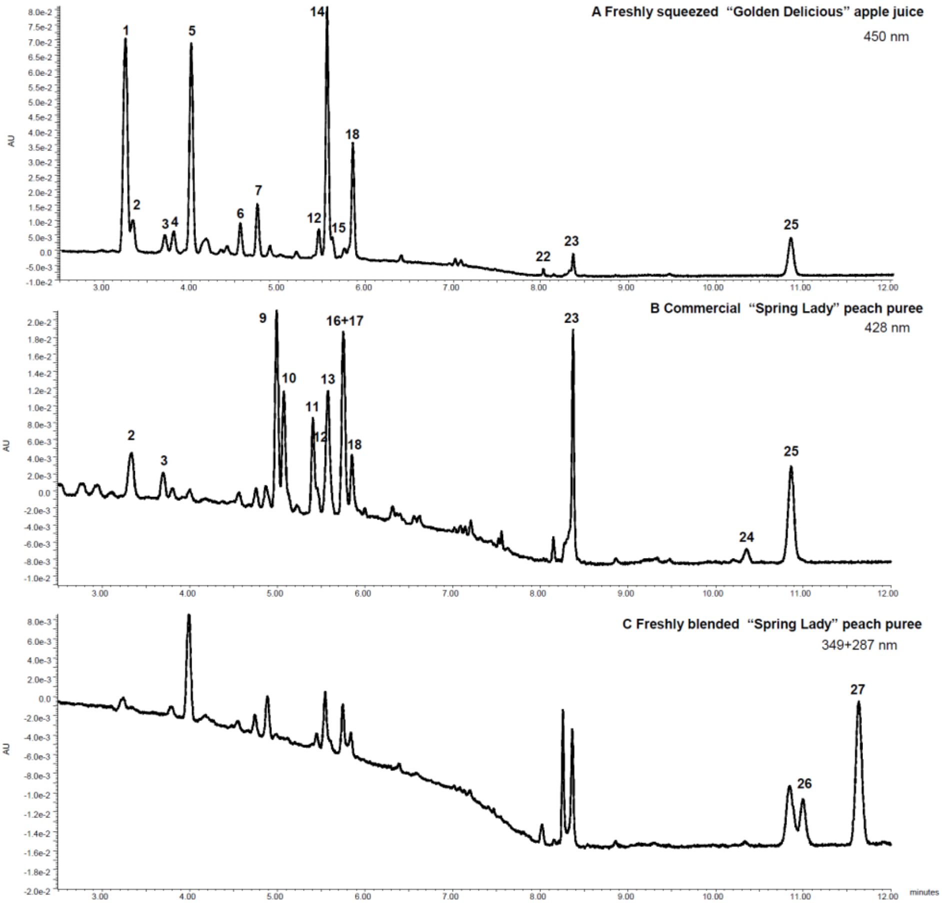


Figure 2. (A) Freshly squeezed Golden Delicious apple juice sample analysed at 450 nm; (B) commercial Spring Lady peach puree sample analysed at 428 nm; and (C) freshly blended Spring Lady peach puree sample analysed at 349 and 287 nm.

Figure 2 (A) shows the identification of (all-*E*)-neoxanthin (chromatographic peak 1), (9′*Z*)-neoxanthin (chromatographic peak 4), (all-*E*)-violaxanthin (chromatographic peak 5) and (9*Z*)-violaxanthin (chromatographic peak 14) in freshly squeezed Golden Delicious apple juice using the criteria mentioned above. We assigned the corresponding chromatographic peaks to these four compounds using commercial standards (9′*Z*)-neoxanthin and (all-*E*)-violaxanthin and literature data [21]. The 9*Z* isomers showed a characteristic hypsochromic shift of 4 nm as compared with all-*E* (see spectra data in Supplementary Data) [47, 48]. This shift allowed us to differentiate (9′Z)-neoxanthin (chromatographic peak 4) and (all-*E*)-

violaxanthin (chromatographic peak 5) from (all-*E*)-neoxanthin (chromatographic peak 1) and (9*Z*)-violaxanthin (chromatographic peak 14), respectively. The protonated molecules $[M+H]^+$ of violaxanthin and neoxanthin obtained by APCI could not be distinguished because they have the same molecular weight. However, these molecules were discernible using the transition 601→167, described by Rivera et al. [46], the intensity of which was much higher for neoxanthin than violaxanthin (Figure 3).

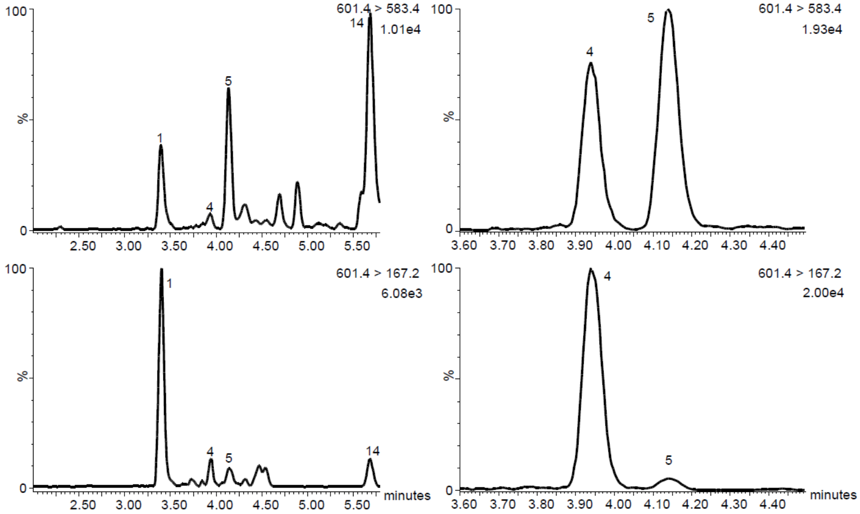


Figure 3. APCI–MS/MS chromatograms from an apple sample (left) and (9′*Z*)-neoxanthin and (all-*E*)-violaxanthin standard carotenoids (right). Transitions 601→583 (top) and 601→167 *m*/*z* (bottom).

5,8-Epoxycarotenoids, together with their 5,6-epoxide isomers, are present in a range of fruit juices [16, 47, 49]. 5,6-Epoxides readily rearrange to form 5,8-epoxides upon treatment with dilute acids, irradiation of the solutions, etc. [6] These epoxide–furanoid rearrangements of violaxanthin, neoxanthin and antheraxanthin are described in Supplementary Data. The presence of 5,8-epoxycarotenoids was confirmed by tandem mass spectrometry (MS/MS) using the same transitions of 5,6-epoxycarotenoids. This allowed us to determine which 5,8-epoxycarotenoids were formed from each 5,6-epoxycarotenoid. Nevertheless, we were unable to distinguish 5,8-epoxycarotenoids from the corresponding 5,6-epoxycarotenoids using MS/MS transitions because they are the same for both groups [47, 48]. As the two epimers have a $\lambda_{max} \approx 20$ nm lower than that in (all-*E*)-neoxanthin in the UV-vis spectrum, the chromatographic peaks 2 and 3 were assigned to the two epimers of neochrome [6, 48]. Because it has not been possible to assign the chiral configuration (8′*R*) or (8′*S*) to these two chromatographic peaks through UV-vis absorption spectra and MS, they have been named as neochrome A and B. These compounds were detected mainly in freshly squeezed apple juice. This finding is consistent with the observation that the apple juices had a higher neoxanthin content than the juice of the other fruits studied. Two epimers for the (all-*E*)-isomer (chromatographic peaks 6 and 7) and two more for the (9*Z*)-isomer (chromatographic peaks 12 and 15) of luteoxanthin were identified in freshly squeezed apple juices and freshly made peach purees (Figure 2 (A) and (B)). The identification was corroborated with the hypsochromic shift of 20 nm, which decreased the longest-wavelength absorption band of all-*E* and 9*Z* to 450 and 444 nm, respectively [47]. As indicated above, the epimers (8′*R*) and (8′*S*) were not assigned to a specific chromatographic peak, consequently these peaks have been named as luteoxanthin A and B. Furthermore, three chromatographic peaks (9, 10 and 13) were identified as the three stereoisomers of auroxanthin on the basis of the presence of two 5,8-furanoid rings. Their presence was supported considering that the maximum of the UV-vis spectrum was at $\lambda_{\text{max}} \approx 40$ nm lower than that of violaxanthin [5, 48]. Following the same criteria for the inability to distinguish stereoisomers of auroxanthin (8*R*, 8′*R*), (8*R*, 8′*S*) and (8*S*, 8′*S*), the chromatographic peaks have been named as auroxanthin A, B and C. These compounds were found mainly in processed juices and purees (Figure 2 (C)). Monoepoxycarotenoid (all-*E*)-antheraxanthin (chromatographic peak 8) was identified using the retention time of a commercial standard. In addition, the chromatographic peaks 11 and 12, at retention times 5.48 and 5.74 min, were tentatively assigned to two epimers of mutatoxanthin. As before for the violaxanthin and neoxanthin isomers, the hypsochromic shift of 20 nm allowed us to identify these 5,8-epoxycarotenoids [49]. (all-*E*)-Antheraxanthin and mutatoxanthin were found in the peach purees. Mutatoxanthin epimers (A and B), like auroxanthin, were found mainly in processed fruit juices and purees (Figure 2 (C)). Similar results have been reported in other matrices [5, 49, 50].

(all-*E*)-Zeaxanthin and (all-*E*)-lutein (chromatographic peaks 17 and 18, respectively) were determined by direct comparison with commercial standards. The identification of the three consecutive peaks located close to 7 min (chromatographic peaks 19, 20 and 21) was achieved using data published by Fu et al. [25], which tentatively identified them as lutein isomers. These isomers were found mainly in pear, which exhibited the highest content of (all-*E*)-lutein. Similarly, the monohydroxycarotenoid region was examined in the chromatogram, identifying β-cryptoxanthin (chromatographic peak 23) at retention time 8.42 min by comparison with a standard. The assignment of α-cryptoxanthin (8.06 min, chromatographic peak 24) was corroborated considering the UV-vis hypsochromic shift of ≈3 nm, its mass spectrum and its chromatographic behaviour [46].

The presence of β-carotene (chromatographic peak 25) was confirmed by co-elution with a commercial standard. Chromatographic peak 23 was assigned to ζ-carotene because of its characteristic UV-vis spectrum (λ_{max} = 426 nm) and chromatographic behaviour relative to the other geometrical isomers of carotene [28, 51, 52]. In contrast to all other carotenoids, phytoene (chromatographic peak 27) and phytofluene (chromatographic peak 26) showed maxima in the UV range. Their identification was based on UV-vis absorption spectra and was confirmed by considering the MS transitions reported by Rivera et al. [46].

3.5. Application of the method developed.

Finally, the method developed was applied to freshly squeezed juices of several apple varieties to assess its capacity to distinguish between monovarietal juices (Table 4). Given that apple has a complex carotenoid profile [53], we considered it a suitable matrix for evaluating the method. The carotenoid profile obtained was very similar to that described previously for fresh fruit [54]. The main carotenoids in freshly squeezed apple juices were (all-*E*)-lutein, β-cryptoxanthin, β-carotene and geometrical isomers of violaxanthin and neoxanthin. Isomers of luteoxanthin and neochrome, (all-*E*)-antheraxanthin and (all-*E*)-α-cryptoxanthin appeared in minor amounts. Luteoxanthin and neochrome isomers theoretically appear from the rearrangement of their respective 5,6-epoxides because of the acidic conditions [6, 16].

For the freshly squeezed juices of the six most popular commercial apple varieties consumed in Catalonia, the content of total carotenoid ranged from 552 μg/100 g to 186 μg/100 g, as determined by UPLC. The Golden Delicious variety had the highest carotenoid concentration and Granny Smith the lowest. However, the latter showed the highest lutein concentration (48.6 μg/100 g) followed by Golden Delicious (36.8 μg/100 g) and Red Delicious (28.4 μg/100 g). Golden Delicious contained 41.6 μg/100 g of β-carotene, whereas in other varieties the amounts of this compound ranged from 14.5 to 22.6 μg/100 g. The highest concentration (19.3 μg/100 g) of β-cryptoxanthin was also observed in this variety. Golden Delicious, Fuji and Royal Gala registered the highest amounts of violaxanthin, whereas the highest concentrations of neoxanthin were found in Golden Delicious and Fuji varieties.

Table 4. Carotenoid content (μg/100 g fresh matter) of freshly squeezed juice from several varieties of apple.

Carotenoid	**Granny Smith** [a]	**Golden Delicious** [a]	**Red Delicious** [a]	**Royal Gala** [a]	**Pink Lady** [a]	**Fuji** [a]
(all-*E*)-Neoxanthin	16.0 ± 1.1	87.2 ± 1.6	28.3 ± .1.6	31.2 ± 0.9	22.9 ± 0.7	67.3 ± 2.0
Neochrome A	9.3 ± 0.1	17.9 ± 0.3	12.1 ± 0.1	11.0 ± 0.1	10.6 ± 0.1	14.2 ± 0.5
Neochrome B	8.3 ± 0.1	12.1 ± 0.1	9.5 ± 0.3	8.6 ± 0.1	8.4 ± 0.1	10.0 ± 0.2
(9′*Z*)-Neoxanthin	11.9 ± 0.4	16.6 ± 0.5	11.7 ± 0.3	10.6 ± 0.0	11.5 ± 0.3	12.9 ± 0.3
(all-*E*)-Violaxanthin	13.4 ± 0.7	99.2 ± 5.8	23.1 ± 1.4	70.8 ± 2.2	35.8 ± 2.0	84.2 ± 3.0
(all-*E*)-Luteoxanthin A	7.6 ± 0.2	17.3 ± 0.4	10.2 ± 0.2	14.6 ± 0.3	12.3 ± 0.3	13.7 ± 0.1
(all-*E*)-Luteoxanthin B	9.1 ± 0.3	23.0 ± 1.1	13.0 ± 0.3	19.8 ± 0.5	16.6 ± 0.7	13.7 ± 0.3
(all-*E*)-Antheraxanthin		11.3 ± 0.3		9.4 ± 0.1		12.1 ± 0.3
(9*Z*)-Luteoxanthin A	6.6 ± 0.2	14.2 ± 0.6	9.1 ± 0.3	16.3 ± 0.3	12.1 ± 0.2	11.6 ± 0.5
(9*Z*)-Violaxanthin	14.2 ± 0.8	134.5 ± 4.1	35.2 ± 0.9	96.6 ± 4.4	55.1 ± 2.2	113.2 ± 4.1
(9*Z*)-Luteoxanthin B	7.1 ± 0.4	12.2 ±0.4	8.8 ± 0.2	13.7 ± 0.3	10.4 ± 0.2	11.2 ± 0.4
(all-*E*)-Lutein	48.6 ± 4.9	36.8 ± 2.2	28.4 ± 0.9	14.9 ± 0.1	18.6 ± 0.2	19.0 ± 0.3
(all-*E*)-α-Cryptoxanthin	7.8 ± 0.0	9.6 ± 0.2	9.4 ± 0.2	11.3 ± 0.2	9.7 ± 0.2	8.7 ± 0.1
(all-*E*)-β-Cryptoxanthin	8.9 ± 0.1	19.4 ± 0.1	12.6 ± 0.4	12.7 ± 0.3	16.3 ± 0.5	13.7 ± 0.3
β-Carotene	17.8 ± 0.3	41.7 ± 3.5	16.6 ± 0.9	14.6 ± 0.9	22.4 ± 1.5	22.7 ± 1.2

Table 5. Carotenoid content (μg/100 g fresh matter) of freshly blended and commercial purees of Miraflores and Spring Lady peaches.

Carotenoid	**Commercial Miraflores peach puree** [a]	**Commercial Spring Lady peach puree** [a]	**Freshly blended Miraflores peach puree** [a]	**Freshly blended Spring Lady peach puree** [a]
(all-*E*)-Neoxanthin			54.0 ± 3.2	53.7 ± 2.0
Neochrome A	42.1 ± 2.4	34.2 ± 3.0		
Neochrome B	29.3 ± 0.7	24.8 ± 1.1		
(9′*Z*)-Neoxanthin			54.8 ± 4.0	42.4 ± 2.0
(all-*E*)-Violaxanthin			703.8 ± 57.4	412.7 ± 54.4
(all-*E*)-Luteoxanthin A	26.1 ± 2.2	16.7 ± 1.1	54.7 ± 4.1	33.1 ± 2.5
(all-*E*)-Luteoxanthin B	27.2 ± 2.96	18.5 ± 1.3	94.4 ± 7.3	60.4 ± 3.6
(all-*E*)-Antheraxanthin			162.1 ± 22.1	84.2 ± 11.8
Auroxanthin A	112.0 ± 10.2	84.7 ± 11.2		
Auroxanthin B	80.5 ± 5.8	60.9 ± 6.6		
Mutatoxanthin A	69.7 ± 5.0	50.3 ± 3.9		
(9*Z*)-Luteoxanthin A	32.9 ± 3.3	23.3 ± 1.2	56.4 ± 4.2	37.7 ± 3.0
Auroxanthin C	88.6 ± 7.4	67.8 ± 6.5		
(9*Z*)-Violaxanthin			190.4 ± 32.3	181.5 ± 29.3
(9*Z*)-Luteoxanthin B	31.3 ± 2.7	22.1 ± 1.3	46.8 ± 3.3	27.5 ± 1.8
Mutatoxanthin B	56.5 ± 4.3	41.2 ± 4.1		
(all-*E*)-Zeaxanthin	87.6 ± 10.6	47.3 ± 3.2	105.5 ± 29.7	43.5 ± 5.6
(all-*E*)-Lutein	55.2 ± 3.6	42.0 ± 2.3	83.9 ± 7.6	49.9 ± 3.7
(all-*E*)-β-Cryptoxanthin	512.0 ± 56.5	166.4 ± 36.5	635.67 ± 79.4	145.9 ± 28.8
ζ-Carotene	78.4 ± 11.4	21.4 ± 12.0	109.8 ± 29.6	19.6 ± 2.4
β-Carotene	226.5 ± 22.2	153.6 ± 12.0	307.8 ± 25.3	165.0 ± 25.4
Phytofluene	34.0 ± 0.9	19.6 ± 0.9	47.8 ± 4.9	17.7 ± 1.1
Phytoene	96.2 ± 8.2	52.4 ± 4.8	119.07 ±18.8	39.9 ± 7.7

[a] Mean and SD corresponding to six batches ($n = 6$).

Once the usefulness of the method to accurately distinguish freshly squeezed juices was confirmed from six apple varieties, we studied the changes in carotenoid profiles in other monovarietal fruit products. For this purpose, we analysed six commercial samples of purees of Miraflores and Spring Lady peaches and Blanquilla and Conference pear juices, which were processed industrially. Fresh, homemade peach puree and pear juice were also analysed. Table 5 shows the concentrations of the carotenoids present in these samples.

In freshly blended peach purees (all-*E*)-violaxanthin was the main carotenoid followed by β-cryptoxanthin and β-carotene. (9*Z*)-Violaxanthin, neoxanthin isomers, luteoxanthin isomers, (all-*E*)-zeaxanthin, (all-*E*)-lutein, (all-*E*)-antheraxanthin, ζ-carotene, phytofluene and phytoene were also found. It is important to note that ζ-carotene was tentatively identified and quantified. This is the first time that this carotene has been reported in peach samples. On the other hand, in the commercial peach purees the main carotenoids were auroxanthin isomers, β-cryptoxanthin and β-carotene. Neochrome isomers, luteoxanthin isomers, (all-*E*)-zeaxanthin, (all-*E*)-lutein, mutatoxanthin isomers, ζ-carotene, phytofluene and phytoene were also found. Figure 4 shows the different profile in the region of the epoxycarotenoids for the commercial and freshly blended Miraflores peach purees. The same was observed for commercial and freshly blended Spring Lady peach purees. These profile differences could be attributed to the processing time and the temperature used for pasteurization that increase isomerization of 5,6- to 5,8-epoxides in the presence of acid media. The content of violaxanthin isomers was higher in the freshly blended puree, whereas the epimers of auroxanthin (derived from violaxanthin isomers) were higher in the commercial juices. Neoxanthin isomers and antheraxanthin rearrangements exhibited the same behaviour, promoting the presence of neochrome epimers and the mutatoxanthin epimers, respectively. Comparing freshly blended purees and commercial purees, the relative ratios of the other carotenoids remained unaltered.

Trying to distinguish between varieties of Spring Lady and Miraflores peach purees, the first evidence was that the total content of carotenoids was higher in Miraflores than in Spring Lady for both freshly blended and commercial purees (Table 5). The presence 5,6-epoxycarotenoids in freshly blended purees of Spring Lady and Miraflores could be used to differentiate between them. Indeed, (9′Z)-neoxanthin, (all-*E*)-violaxanthin and (all-*E*)-antheraxanthin, whose respective amounts were 54.8, 703.8 and 162.1 μg/100 g in the Spring Lady variety, were in contrast to the lower values of the Miraflores variety 42.4, 412.7 and 84.2 μg/100 g, respectively. In commercial peach purees the 5,8-epoxycarotenoids allow us to differentiate the Spring Lady and Miraflores varieties. In this case, the contents of 71.4 μg/100 g of neochrome (A and B), 281.1 μg/100 g of auroxanthin (A, B and

C) and 126.2 μg/100 g of mutatoxanthin (A and B) were higher than the contents of the Miraflores variety 59.0, 213.4 and 91.5 μg/100 g, respectively.

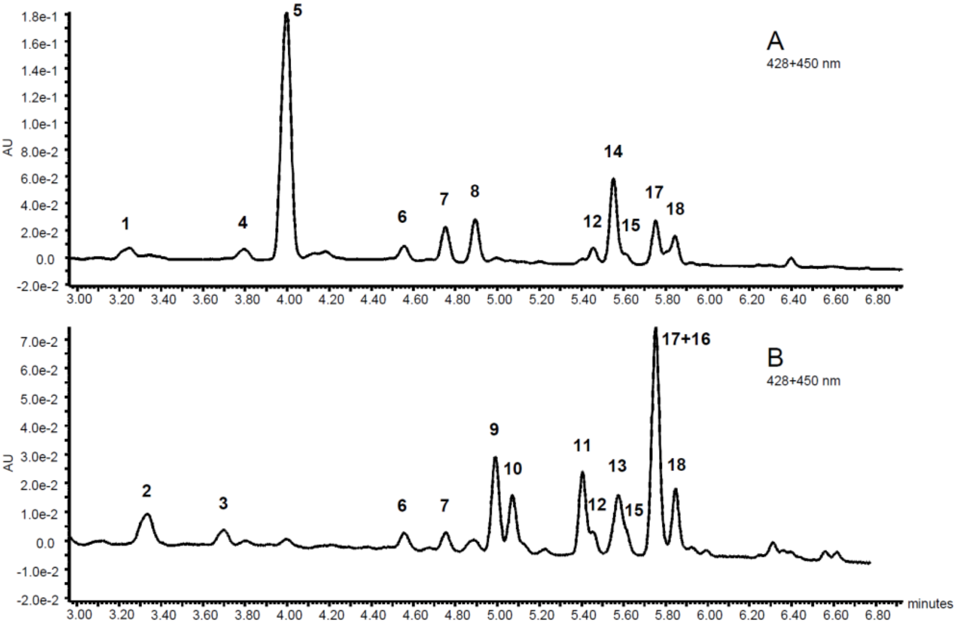


Figure 4. Carotenoid profile over the range 3.00–6.90 min at 428 + 450 nm for freshly blended (A) and commercial (B) Miraflores peach puree.

As was noted above, the hydroxycarotenoid and carotene profiles were quite similar between freshly blended and commercial purees. In this case, the contents of β-cryptoxanthin were much higher in the Miraflores than in the Spring Lady peaches, whereas the β-carotene content was not so different. The concentrations of (all-*E*)-zeaxanthin and phytoene were also higher in Miraflores peaches.

The same methodology was applied to pear juices, which showed a simpler carotenoid profile, as already described [55, 56]. The profile was dominated mainly by (all-*E*)-lutein followed by β-carotene (Table 6). The epoxycarotenoid content of this kind of fruit juice has not been described previously. Our finding might be due to the higher sensitivity achieved using UPLC, which allowed the identification and quantification of isomers of neoxanthin and violaxanthin in the freshly squeezed pear juices. Of note, pear juice was the only sample of the study in which lutein isomers were sufficient to be quantified.

Table 6. Carotenoid content (μg/100 g fresh matter) of freshly squeezed and commercial juices of Conference and Blanquilla pears.

Carotenoid	**Commercial Conference pear juice** [a]	**Commercial Blanquilla pear juice** [a]	**Freshly squeezed Conference pear juice** [a]	**Freshly squeezed Blanquilla pear juice** [a]
(all-*E*)-Neoxanthin			2.6 ± 0.3	3.3 ± 0.1
Neochrome A	1.6 ± 0.1	1.3 ± 0.0	2.8 ± 0.2	2.4 ± 0.1
Neochrome B	1.3 ± 0.0	1.2 ± 0.0	2.5 ± 0.2	2.2 ± 0.1
(9′*Z*)-Neoxanthin			2.3 ± 0.1	2.5 ± 0.1
(all-*E*)-Violaxanthin			2.3 ± 0.2	2.6 ± 0.3
(all-*E*)-Luteoxanthin A			2.0 ± 0.0	2.2 ± 0.1
(all-*E*)-Luteoxanthin B			2.2 ± 0.1	2.3 ± 0.2
(all-*E*)-Antheraxanthin			2.7 ± 0.1	3.2 ± 0.2
(9*Z*)-Violaxanthin			2.2 ± 0.1	2.4 ± 0.1
(all-*E*)-Zeaxanthin	1.6 ± 0.1	2.0 ± 0.2	3.0 ± 0.2	4.9 ± 0.4
(all-*E*)-Lutein	16.8 ± 2.7	8.7 ± 1.5	36.6 ± 10.4	35.9 ± 6.8
Lutein isomers	2.6 ± 0.2	2.3 ± 0.1	2.1 ± 0.4	1.9 ± 0.4
(all-*E*)-β-Cryptoxanthin	2.8 ± 0.3	2.0 ± 0.2	3.8 ± 0.4	3.6 ± 0.4
ζ-Carotene	1.2 ± 0.1			
β-Carotene	5.7 ± 0.8	2.9 ± 0.5	13.1 ± 2.8	16.8 ± 3.7
Phytoene	1.1 ± 0.0	0.8 ± 0.0	1.8 ± 0.3	

[a] Mean and SD corresponding to six batches (*n* = 6).

In the pear juices, the hydroxycarotenoids and carotenes identified were the same in freshly squeezed and commercial juice, but in this case, the profile was different. These results could be a consequence of the difficulty of reproducing the commercial juice process in the lab. Despite this fact, to distinguish between varieties of Conference and Blanquilla the ratio between (all-*E*)-lutein and (all-*E*)-zeaxanthin could be used. This ratio was higher in the Blanquilla than in the Conference variety in both freshly squeezed and commercial juices.

4. Conclusions.

Here, we described a UPLC–PDA–MS/MS analytical method that allows the simultaneous determination of epoxycarotenoids, hydroxycarotenoids and carotenes in monovarietal freshly made and industrially processed fruit juices. Extraction of carotenoids was performed using freeze-dried samples to reduce time and solvent consumption. The extraction using methanol as the first extracting solvent provided the highest extraction yield for all carotenoids. The recoveries of the whole extraction method were over 90% and repeatability was better than 10% (%RSD, $n = 3$).

Separation of the main epoxycarotenoids, hydroxycarotenoids and carotenes was achieved using common solvents in UPLC (ACN, methanol and water) in less than 17 min. The chromatographic separation was focused on epoxycarotenoids, which presented the profile of greatest complexity in fruit juices. The method allowed the proper separation of the main 5,6- and 5,8-epoxycarotenoids and the separation of the geometrical isomers and epimers for the first time in a UPLC system. Up to 27 carotenoids were tentatively identified in apple, peach and pear products, based on chromatographic retention time, UV-vis absorption spectra and MS/MS.

The developed method allowed us to observe that the six samples of apple varieties show a different carotenoid profile. These results show that the carotenoid analysis could be useful to differentiate between apple varieties. The method was applied to study the changes in the carotenoid profile in commercial and freshly made monovarietal fruit products (peach purees and pear juices). We found that the 5,6-epoxycarotenoids are the main carotenoids defining the profile in the freshly made samples, while they did not appear in the commercial samples. The rearrangements of 5,6-epoxycarotenoids to 5,8-epoxycarotenoids during the corresponding industrial processes might explain these results. The relative ratios of the other carotenoids remained unaltered when comparing freshly blended and commercial products.

Acknowledgement.

The authors would like to thank Zucasa for providing samples. We also thank Tania Yates for the proofreading of the manuscript.

References.

[1] P. Astorg, Trends Food Science and Technology, 8 (1997) 406.

[2] N.I. Krinsky, Free Radical Biology & Medicine, 7 (1989) 617.

[3] A. Oliveira, M. Pintado, D.P.F. Almeida, LWT- Food Science and Technology, 49 (2012) 202.

[4] A. Rawson, A. Patras, B.K. Tiwari, F. Noci, T. Koutchma, N. Brunton, Food Research International., 44 (2011) 1875.

[5] A.J. Meléndez-Martínez, I.M. Vicario, F.J. Heredia, Journal of Food Composition and Analysis, 22 (2009) 295.

[6] A. Asai, M. Terasaki, A. Nagao, Journal of Nutrition, 134 (2004) 2237.

[7] K.H. Cha, H.J. Lee, S.Y. Koo, D.-G. Song, D.-U. Lee, C.-H. Pan, Journal of Agricultural and Food Chemistry, 58 (2009) 793.

[8] M. Herrero, L. Jaime, P.J. Martín-Álvarez, A. Cifuentes, E. Ibáñez, Journal of Agricultural and Food Chemistry, 54 (2006) 5597.

[9] M.M. Sanagi, H.H. See, W.A.W. Ibrahim, A.A. Naim, Analytica Chimica Acta, 538 (2005) 71.

[10] E. Cadoni, M. Rita De Giorgi, E. Medda, G. Poma, Dyes Pigment., 44 (1999) 27.

[11] S. Machmudah, Y. Kawahito, M. Sasaki, M. Goto, Journal of Supercritical Fluids, 44 (2008) 308.

[12] G. Panfili, A. Fratianni, M. Irano, Journal of Agricultural and Food Chemistry, 52 (2004) 6373.

[13] M.G. Dias, L. Oliveira, M.F.G.F.C. Camões, B. Nunes, P. Versloot, P.J.M. Hulshof, Journal of Chromatography A, 1217 (2010) 3494.

[14] 1990. AOAC methods of analysis. 16th Ed. Arlington

[15] A.K. Taungbodhitham, G.P. Jones, M.L. Wahlqvist, D.R. Briggs, Food Chemistry, 63 (1998) 577.

[16] A.J. Meléndez-Martínez, I.M. Vicario, F.J. Heredia, Food Chemistry, 104 (2007) 169.

[17] A.J. Meléndez-Martínez, I.M. Vicario, F.J. Heredia, Journal of Food Composition and Analysis, 20 (2007) 638.

[18] R.B. Assunção, A.Z. Mercadante, Journal of Food Composition and Analysis, 16 (2003) 647.

[19] C. Kurz, R. Carle, A. Schieber, Food Chemistry, 110 (2008) 522.

[20] M.G. Dias, M.F.G.F.C. Camões, L. Oliveira, Food Chemistry, 113 (2009) 808.

[21] P. Molnár, M. Kawase, K. Satoh, Y. Sohara, T. Tanaka, S. Tani, H. Sakagami, H. Nakashima, N. Motohashi, N. Gyémánt, J. Molnár, Phytotherapy Research, 19 (2005) 700.

[22] P. Dugo, V. Škeříková, T. Kumm, A. Trozzi, P. Jandera, L. Mondello, Analytical Chemistry, 78 (2006) 7743.

[23] P. Dugo, M. Herrero, T. Kumm, D. Giuffrida, G. Dugo, L. Mondello, Journal of Chromatography A, 1189 (2008) 196.

[24] B. Chauveau-Duriot, M. Doreau, P. Nozière, B. Graulet, Analytical and Bioanalytical Chemistry, 397 (2010) 777.

[25] W. Fu, M. Magnúsdóttir, S. Brynjólfson, B. Palsson, G. Paglia, Analytical and Bioanalytical Chemistry, 404 (2012) 3145.

[26] I. Guzman, G.G. Yousef, A.F. Brown, Journal of Agricultural and Food Chemistry, 60 (2012) 7238.

[27] D. Bohoyo-Gil, D. Dominguez-Valhondo, J.J. García-Parra, D. González-Gómez, Journal of Food Composition and Analysis, 235 (2012) 1055.

[28] G. Britton, S. Liaaen-Jensen, H. Pfander, 2004.

[29] P.D. Fraser, M.E.S. Pinto, D.E. Holloway, P.M. Bramley, Plant Biotechnology Journal, 24 (2000) 551.

[30] D.J. Hart, K.J. Scott, Food Chemistry, 54 (1995) 101.

[31] B. Simonovska, I. Vovk, V. Glavnik, K. Černelič, Journal of Chromatography A, 1276 (2012) 95.

[32] Cortés, M.J. Esteve, A. Frígola, F. Torregrosa, Journal of Agricultural and Food Chemistry, 52 (2004) 2203.

[33] S. Rivera, R. Canela, Molecules, 17 (2012) 11255.

[34] S. Georgé, F. Tourniaire, H. Gautier, P. Goupy, E. Rock, C. Caris-Veyrat, Food Chemistry, 124 (2011) 1603.

[35] I. Çinar, LWT- Food Science and Technology, 37 (2004) 363.

[36] Y.C. Tang, B.H. Chen, Food Chemistry, 69 (2000) 11.

[37] P.W. Simon, X.Y. Wolff, Journal of Agricultural and Food Chemistry, 35 (1987) 1017.

[38] N. Harnkarnsujarit, S. Charoenrein, Food Research International, 44 (2011) 3188.

[39] G. Leach, G. Oliveira, R. Morais, Journal of Agricultural and Food Chemistry, 76 (1998) 298.

[40] N.M. Shofian, A.A. Hamid, A. Osman, N. Saari, F. Anwar, M.S. Dek, M.R. Hairuddin, International Journal of Molecular Sciences, 12 (2011) 4678.

[41] J.P. Chen, C.Y. Tai, B.H. Chen, Food Chemistry, 100 (2007) 1005.

[42] D.B. Rodriguez-Amaya, (2001).

[43] Waters Corporation, 34 Maple Street. Milford, MA 01757.

[44] D. Giuffrida, A. Pintea, P. Dugo, G. Torre, R.M. Pop, L. Mondello, Phytochemical Analysis, 23 (2012) 267.

[45] B.S. Inbaraj, H. Lu, C.F. Hung, W.B. Wu, C.L. Lin, B.H. Chen, Journal of Pharmaceutical and Biomedical Analysis, 47 (2008) 812.

[46] S. Rivera, F. Vilaró, R. Canela, Analytical and Bioanalytical Chemistry, 400 (2011) 1339.

[47] V.V. de Rosso, A.Z. Mercadante, Journal of Agricultural and Food Chemistry, 55 (2007) 5062.

[48] P. Crupi, R.A. Milella, D. Antonacci, Journal of Mass Spectrometry, 45 (2010) 971.

[49] A.J. Meléndez-Martínez, G. Britton, I.M. Vicario, F.J. Heredia, Food Research International, 38 (2005) 931.

[50] A.Z. Mercadante, D.B. Rodriguez-Amaya, Journal of Agricultural and Food Chemistry, 46 (1998) 128.

[51] M. Bononi, I. Commissati, E. Lubian, A. Fossati, F. Tateo, Analytical and Bioanalytical Chemistry, 372 (2002) 401.

[52] W. Stahl, A.R. Sundquist, M. Hanusch, W. Schwarz, H. Sies, Clinical Chemistry, 39 (1993) 810.

[53] P. Molnár, J. Deli, T. Tanaka, Y. Kann, S. Tani, N. Gyémánt, J. Molnár, M. Kawase, Phytother. Res., 24 (2010) 644.

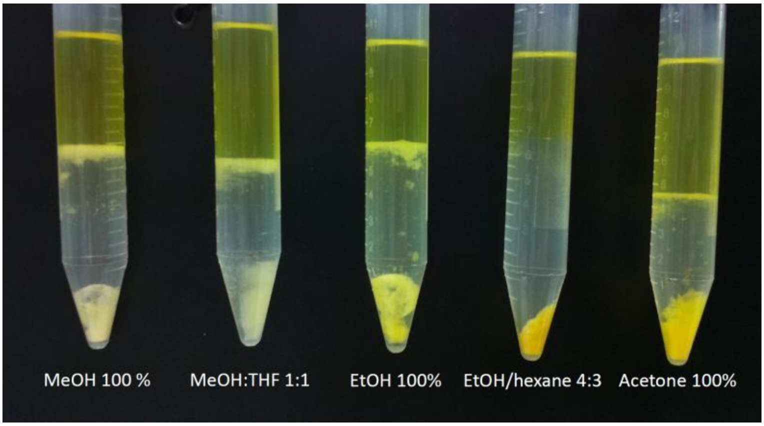
[54] C. Ampomah-Dwamena, S. Dejnoprat, D. Lewis, P. Sutherland, R.K. Volz, A.C. Allan, Journal of Experimental Botany, 63 (2012) 4497.

[55] M. Caller, G. Mackinney, Journal of Food Science., 30 (1965) 393.

[56] M.E. Jaramillo-Flores, L. González-Cruz, M. Cornejo-Mazón, L. Dorantes-Alvarez, G.F. Gutiérrez-López, H. Hernández-Sánchez, Food Science and Technology International, 9 (2003) 271.

APPENDIX A. Supplementary data.

Starting solvent comparison.



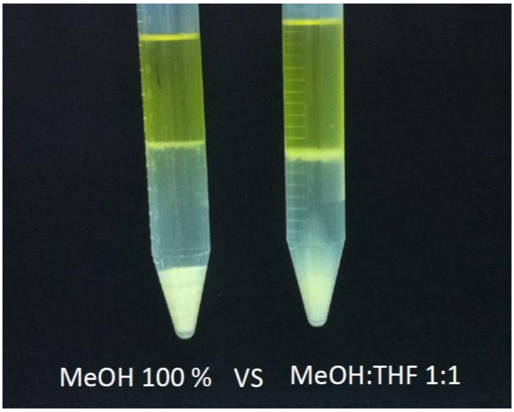


Figure S-1. Picture/Visual comparison of the five starting extraction solvents assessed.

Comparison HSS-T3 vs BEH C18.

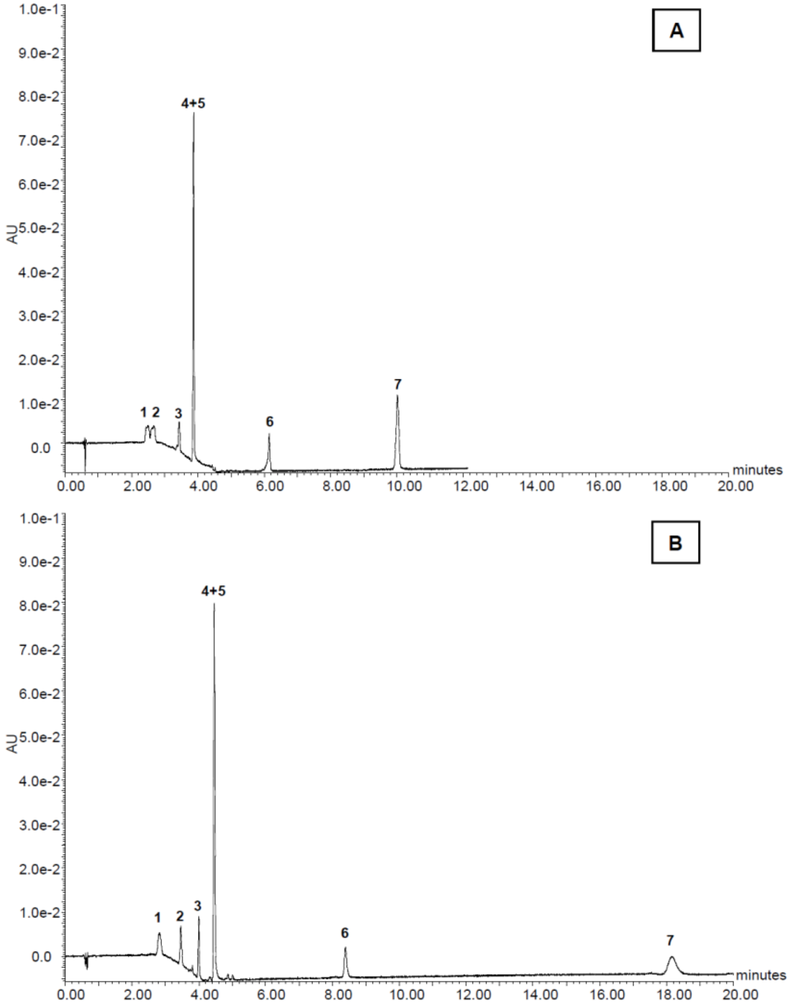


Figure S-2. Comparison of chromatographic separation of standard, carried out with (A) Acquity UHPLC BEH C18 and (B) Acquity UHPLC HSS T3. 1- (9'Z)-neoxanthin, 2- (all-E)-violaxanthin, 3-(all-E)-antheraxanthin, 4- (all-E)-zeaxanthin, 5- (all-E)-lutein, 6-β-cryptoxanthin, 7- β-carotene.

Method HPLC-PDA using YMC 30 column.

The HPLC system consisted of a 2690 Alliance Waters quaternary pump. The detection was performed with a PDA detector Waters.

The chromatographic system was a quaternary pump system Waters Alliance 2695 coupled to PDA detector. The separation was carried out with an HPLC column YMC C30 RP (100 x 2,0 mm i.d., 3 μm) (Wilmington, MA, USA).

Table S-1. HPLC-PDA conditions.

Column:	YMC C30 RP (100 x 2,0 mm i.d., 3 μm) (Wilmington, MA, USA)			
Solvents:	Solvent A: Methanol Solvent B: Mehyll tert-butyl ether Solvent C: Water			
Gradient:	Time (min)	% Solvent A	% Solvent B	% Solvent C
	Initial	93	0	7
	6.11	93	0	7
	7.78	100	0	-
	13.89	70	30	-
	15.56	50	50	-
	17.22	50	50	-
	17.78	30	70	-
	19.44	initial	0	initial
	26.56	initial	0	initial
Flow rate:	0,25 Ml/min			
Column temperature:	25 °C			
Injection volume:	5 μL			

Epoxide–furanoid rearrangements of violaxanthin, neoxanthin.

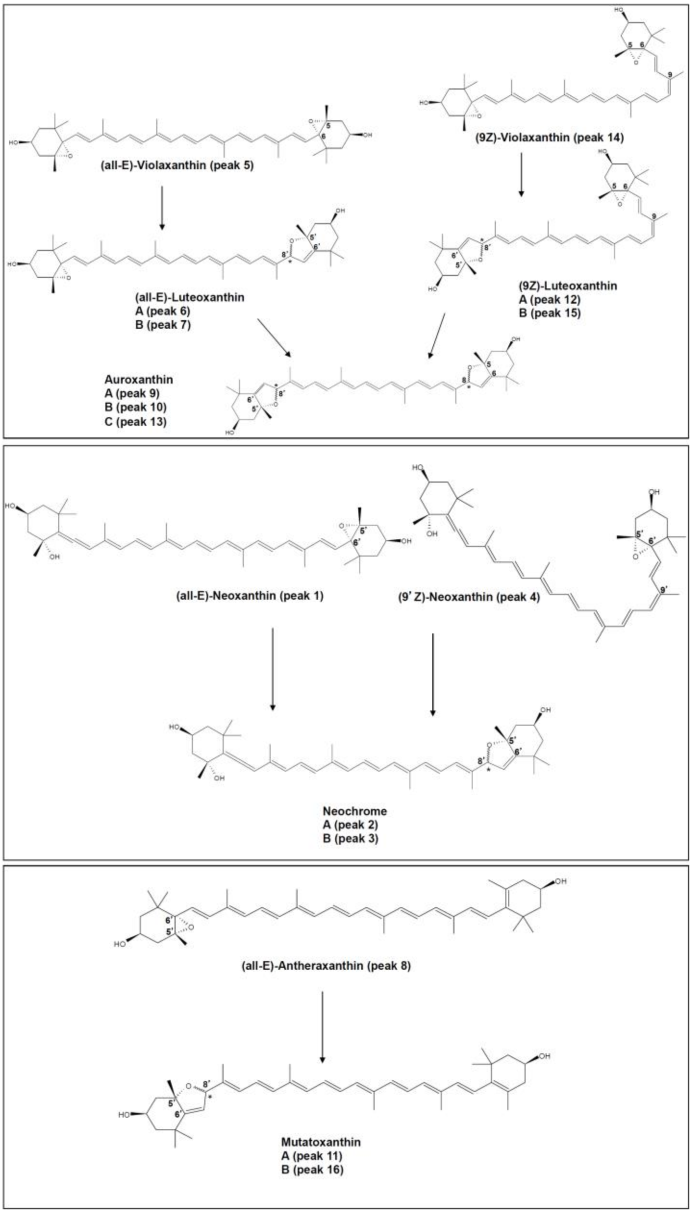


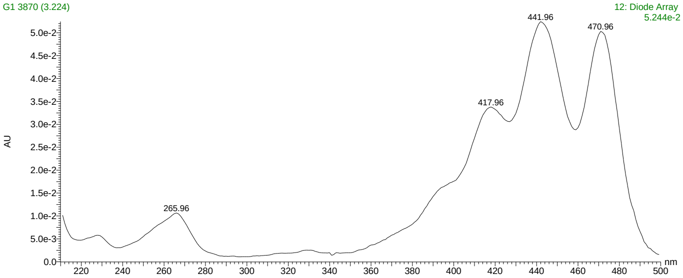
Figure S-3. Scheme of epoxide–furanoid rearrangements of violaxanthin, neoxanthin and antheraxanthin observed in the samples.

UV/Vis-Spectra of the carotenoids.

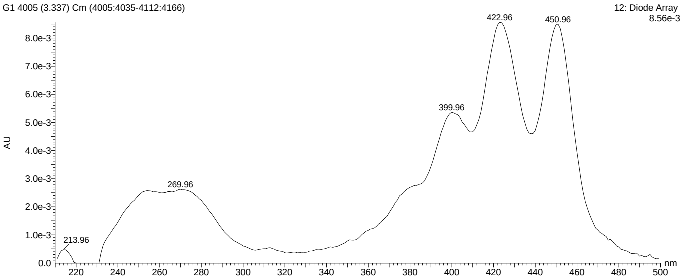
Table S-2. Chromatography and UV-vis parameters of carotenoids found in the samples.

Peak	Carotenoid	$t_{r\ (min)}$	λ_{max} (nm)	%(III/II)[a]
1	(all-*E*)-Neoxanthin	3.24	418;442;471	90
2	Neochrome A	3.33	400;423;451	95
3	Neochrome B	3.70	399;423;451	95
4	(9′*Z*)-Neoxanthin	3.79	329;414;438;466	89
5	(all-*E*)-Violaxanthin	4.00	417;442;471	93
6	(all-*E*)-Luteoxanthin A	4.55	401;424;450	98
7	(all-*E*)-Luteoxanthin B	4.75	401;424;450	98
8	(all-*E*)-Antheraxanthin	4.89	422;448;475	62
9	Auroxanthin A	4.99	384;403;428	100
10	Auroxanthin B	5.07	384;403;428	100
11	Mutatoxanthin A	5.41	408;430;456	60
12	(9*Z*)-Luteoxanthin A	5.46	313;396;418;445	98
13	Auroxanthin C	5.58	384;403;428	100
14	(9*Z*)-Violaxanthin	5.55	328;414;437;466	92
15	(9*Z*)-Luteoxanthin B	5.61	313;397;419;444	98
16	Mutatoxanthin B	5.74	408;430;456	60
17	(all-*E*)-Zeaxanthin	5.76	428;453;478	19
18	(all-*E*)-Lutein	5.85	424;447;475	61
19	Lutein isomer	6.96	332;419;449;471	
20	Lutein isomer	7.02	332;419;449;468	
21	Lutein isomer	7.08	332;419;449;469	36
22	(all-*E*)-α-Cryptoxanthin	8.03	420;449;476	59
23	(all-*E*)-β-Cryptoxanthin	8.37	424;452;479	18
24	ζ-Carotene	10.35	381;402;426	94
25	β-Carotene	10.86	422;452;479	11
26	Phytofluene	11.00	332;349;369	84
27	Phytoene	11.66	275;287;299	

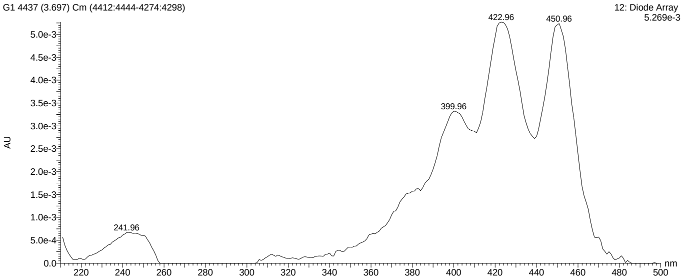
Peak 1



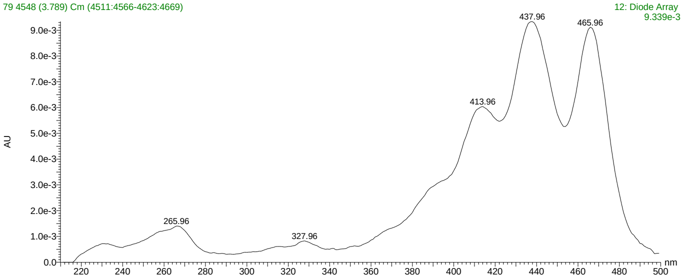
Peak 2



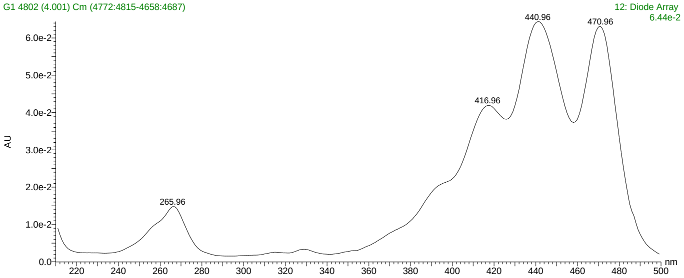
Peak 3



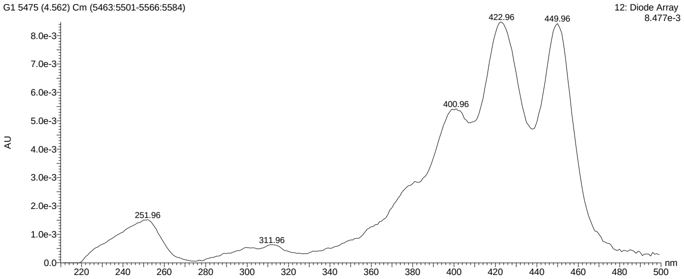
Peak 4



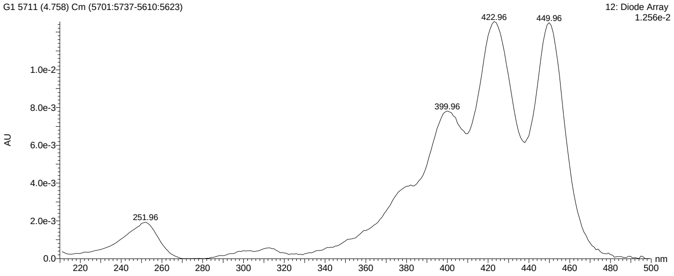
Peak 5



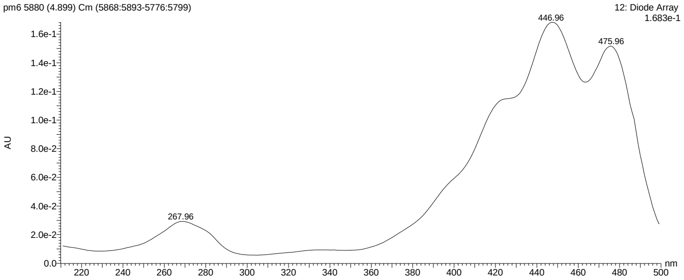
Peak 6



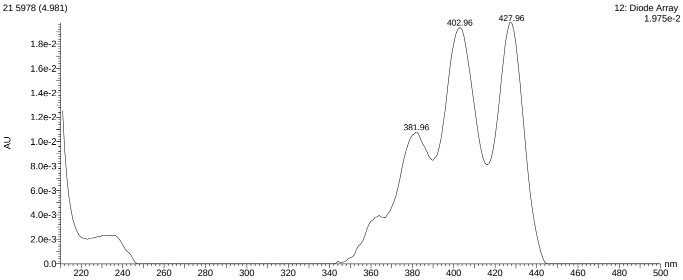
Peak 7



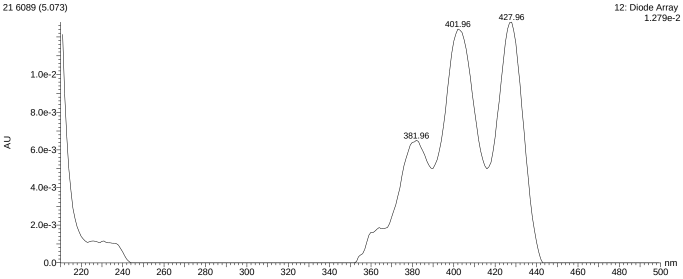
Peak 8



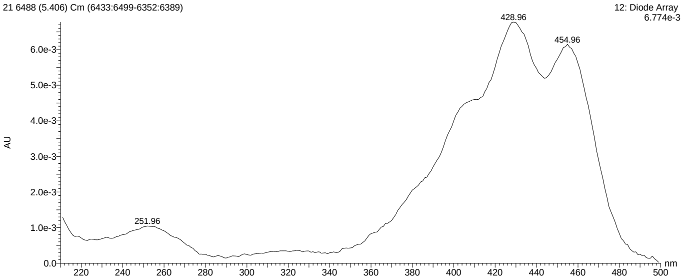
Peak 9



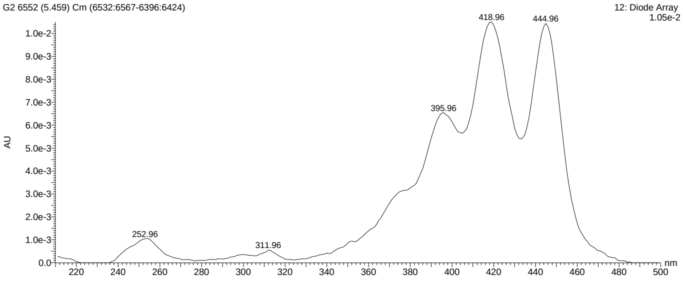
Peak 10



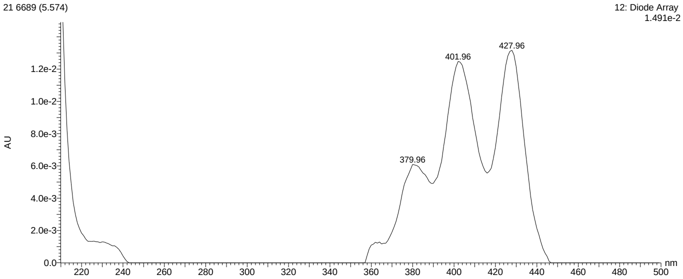
Peak 11



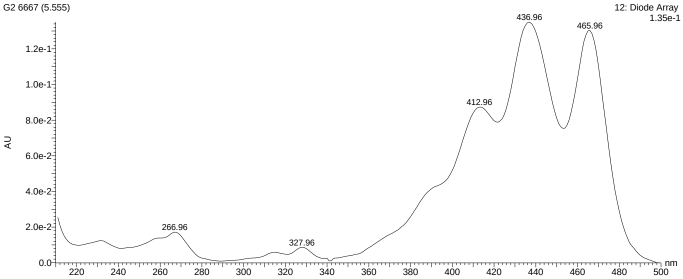
Peak 12



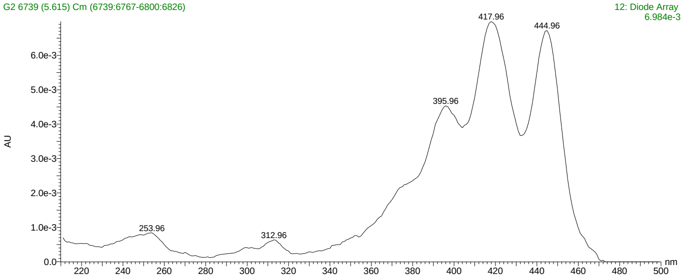
Peak 13



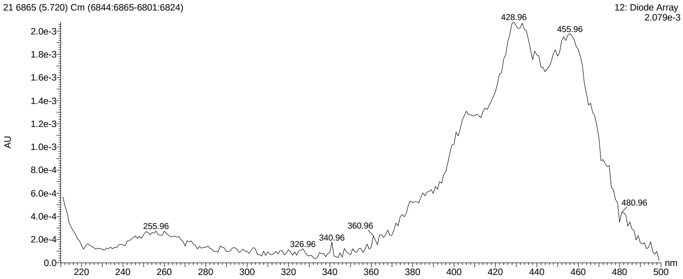
Peak 14



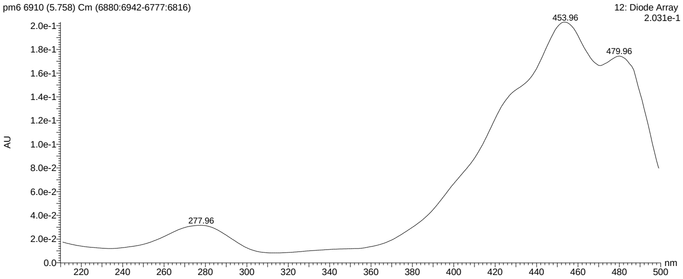
Peak 15



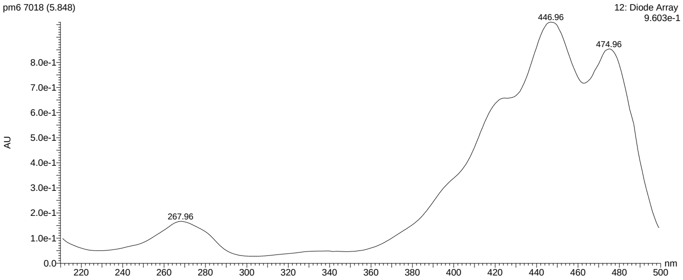
Peak16



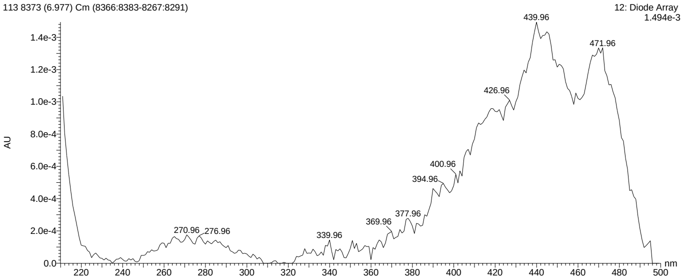
Peak 17



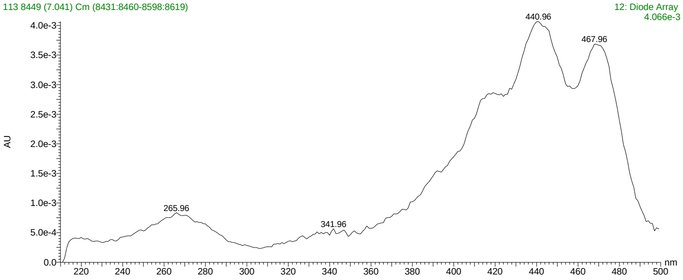
Peak 18



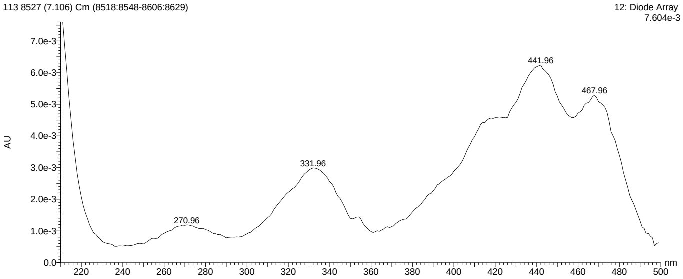
Peak 19



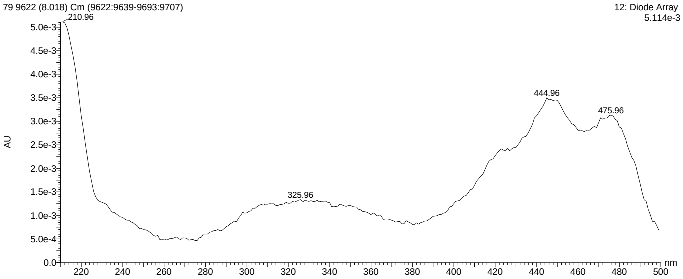
Peak 20



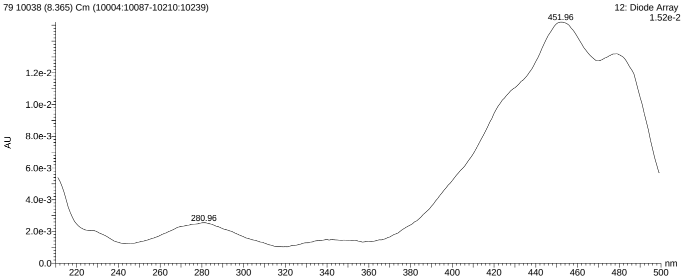
Peak 21



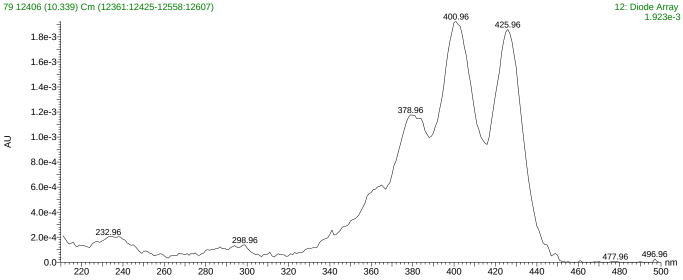
Peak 22



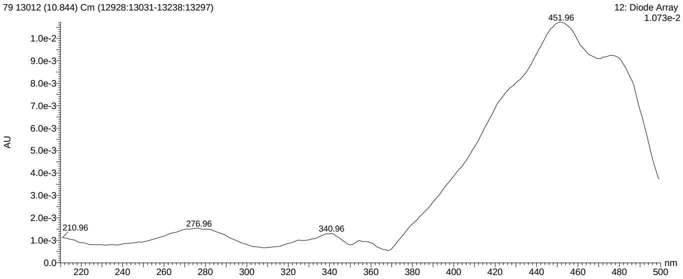
Peak 23



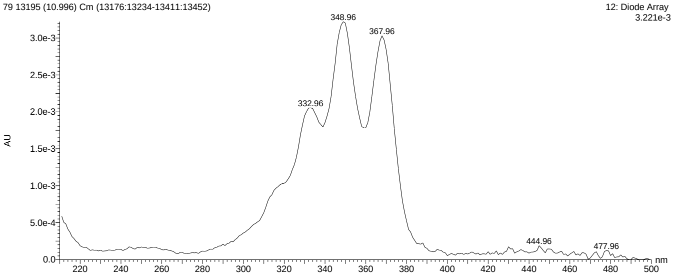
Peak 24



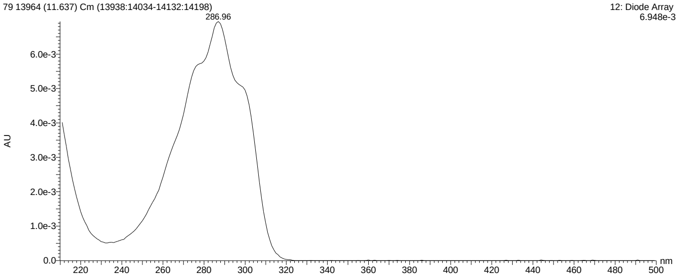
Peak25



Peak 26



Peak 27



Wavelength used in quantification with PDA detector to each carotenoid compound.

Table S-3. Summary of the wavelength and the standard (calibration curve) used to quantify each carotenoid.

Peak	Carotenoid	$t_{r\ (min)}$	$\lambda_{quantitation}$(nm)	Standard used for quantiation
1	(all-*E*)-Neoxanthin	3.24	468	(9’Z)-Neoxanthin
2	Neochrome A	3.33	450	(9’Z)-Neoxanthin
3	Neochrome B	3.70	450	(9’Z)-Neoxanthin
4	(9′*Z*)-Neoxanthin	3.79	468	(9’Z)-Neoxanthin
5	(all-*E*)-Violaxanthin	4.00	468	(all-E)-Violaxanthin
6	(all-*E*)-Luteoxanthin A	4.55	450	(all-E)-Violaxanthin
7	(all-*E*)-Luteoxanthin B	4.75	450	(all-E)-Violaxanthin
8	(all-*E*)-Antheraxanthin	4.89	450	(all-E)-Antheraxanthin
9	Auroxanthin A	4.99	428	(all-E)-Violaxanthin
10	Auroxanthin B	5.07	428	(all-E)-Violaxanthin
11	Mutatoxanthin A	5.41	450	(all-E)-Antheraxanthin
12	(9*Z*)-Luteoxanthin A	5.46	445	(all-E)-Violaxanthin
13	Auroxanthin C	5.58	428	(all-E)-Violaxanthin
14	(9*Z*)-Violaxanthin	5.55	468	(all-E)-Violaxanthin
15	(9*Z*)-Luteoxanthin B	5.61	445	(all-E)-Violaxanthin
16	Mutatoxanthin B	5.74	450	(all-E)-Antheraxanthin
17	(all-*E*)-Zeaxanthin	5.76	478	(all-E)-Zeaxanthin
18	(all-*E*)-Lutein	5.85	478	(all-E)-Lutein
19	Lutein isomer		471	(all-E)-Lutein
20	Lutein isomer		471	(all-E)-Lutein
21	Lutein isomer		471	(all-E)-Lutein
22	(all-*E*)-α-Cryptoxanthin	8.03	450	(all-E)-β-Cryptoxanthin
23	(all-*E*)-β-Cryptoxanthin	8.37	450	(all-E)-β-Cryptoxanthin
24	ζ-Carotene	10.35	426	β-Carotene
25	β-Carotene	10.86	450	β-Carotene
26	Phytofluene	11.00	349	Phytoene
27	Phytoene	11.66	287	Phytoene

3.1.2. A fast and reliable UPLC method to assess the fate of chlorophylls in processed vegetable products.

A FAST AND RELIABLE UPLC METHOD TO ASSESS THE FATE OF CHLOROPHYLLS IN PROCESSED VEGETABLE PRODUCTS.

Antoni Delpino-Rius [a], Diana Cosovanu [a], Jordi Eras [a,b,*], Francisca Vilaró [b], Mercè Balcells [a,*,] Ramon Canela-Garayoa

[a] Chemistry Department, ETSEA, University of Lleida, 25198-Lleida, Spain

[b] Scientific Technical Services, University of Lleida, 25198-Lleida, Spain

Submitted to *Journal of Chromatography*

Keywords: Carotenoid profile; UPLC; Monovarietal fruit juice; Epoxycarotenoid; Hydroxycarotenoid.

ABSTRACT

Chlophyll and their derivatives were successfully determined in tea and processed vegetable and fruit foodstuffs by UPLC with photodiode-array and mass spectrometry detection. Up to 48 chlorophylls and derivatives were identified, the method allowed the proper separation of the chlorophyll derivatives obtained from demetalitation, dephytilation, decarbomethoxylation epimerisation and copperisation in less than 12 min, using an optimised ternary gradient (MeOH, iPrOH,MeCN and H_2O 10 mM of ammonium acetate) on ACQUITY HSS T3 column. Mass spectrometry was used as identification tool applying both ESI and APCI ionisation sources. The method was applied to evaluate the degree of processing in teas of different origin and quality, allowing differentiate between supermarket own-brand tea bags and offered teas by specialised shops. In processed green vegetable and fruit products were determined mainly pheophytins, pheophorbides and pyro derivatives thereof. However, in several products were also detected chlorophyll derivatives food colourants such as: Cu-chlorophyllins, Cu-pheophytins, Cu-pyropheophytins, Cu-pheophorbides and Cu-pyropheophorbides.

1. Introduction.

Chlorophylls belong to the group of the most important bioorganic molecules, because of their important functions in photosynthesis and plant physiology. Chlorophyll compounds fulfil certain biological functions that are effective as long as chlorophyll derivative conserves the basic porphyrin ring structure. Recently, chlorophyll and its derivatives exhibited important health-promoting functions such as antimutagenic and anticarcinogenic activities [1-4]. In the human diet, these compounds are present in many foodstuffs of plant origin. It has been related that chlorophylls are susceptible to chemical and physical changes during the processing [5]. Mild processing leads to pheophytinization reaction, resulting from a generalised liberation of acids that provokes a demetalitation. Demetalitation and epimerisation are also promoted during heat treatment and an additional decarbomethoxylation when the heating time is delayed, originating mainly pyropheophytin [6, 7]. The processing conditions can also induce the enzymatically endogenous activity, arising dephytylated chlorophyll derivatives, mainly pheophorbide, and chlorophyllides at high pH conditions [8]. Pheophorbide can be degraded via pheophytins and via chlorophyllides and literature has related that pheophorbides is the last chromophore derivate involved in the chlorophyll degradation pathway [9]. Storage conditions are also factors to be considered, for instance depending on the light or dark conditions, the reactions of degradation of chlorophylls are different as well as their degradation products [10]. Demetalitation chlorophyll derivatives, which are the main found in processed foods, exhibit a brown olive colour less pleasurable than the bright green of the chlorophylls. It makes that to improve product presentation the food colouring known as E-141(i) and E-141(ii) are usually added. Both are a mixture of a large number of compounds referred as copper complexes of chlorophylls (E-141(i)) and chlorophyllins (E-141(ii)). Thus in food science chlorophylls and their derivatives are used as markers in food processing. In this context, rapid analytical methods are needed to identify and quantify chlorophyll derivatives in processed foodstuff and to track chlorophyll degradation. The study chlorophyll derivatives profile would allow assessment of processing level as well as if it has been added colorant.

To date, reversed-phase high-performance liquid chromatography (HPLC), mainly with C18 but, also C30 stationary phases, has been the principal tool used for the analysis of chlorophyll derivatives in various vegetables matrices. [11-16]. To analyse colorants from copper complexes, C30 and C18 were also used to carry out the separation[17, 18]. In the cases of acidic pigments analysis such as chlorophyllides, pheophorbides and chlorins must be used very high concentration of ion-pairing reagents and is also necessary the use of apolar solvents to elute the more apolar chlorophyll derivatives. The main consequence is that the chromatographic system has to operate in extreme conditions. Recently, ultra performance liquid chromatography (UPLC) technology has also been used to analyse compounds with similar polarity such as carotenoids [19]. This technology offers many advantages over standard HPLC, including reduced run time and less solvent consumption. Although, in the literature there are few articles where this methodology is used to separate chlorophyll derivatives, and only were applied to the separation of phytylated compounds [20].

Here, we sought to develop a rapid and reliable analytical method to determine the chlorophyll derivatives profile in green processed foodstuff from very different plant source such as fruit, vegetable and tea. With this in mind, an UPLC method was developed to obtain a fast separation for the simultaneous determination of chlorophyll derivatives metalited and demetalited as well as phytylated and dephytylated, including Cu^{2+} complexes of chlorophylls (E-141(i)) and copper complexes of chlorophyllins (E-141(ii)). To identify chlorophyll derivatives commercial standards, when were available, were applied to achieve identification. Therefore, ultraviolet–visible (UV-vis), fine structure, mass spectra and published information on retention times in distinct chromatographic systems are also useful for this purpose. Although mass spectrometry (MS) cannot distinguish stereoisomers, this technique is considered reliable for the assignment of chlorophyll derivatives structures. In addition, we used the different ionisation modes and acquisition modes of a tandem mass spectrometer to confirm the molecular mass and the characteristic fragmentation patterns of the chlorophyll derivatives tentatively identified.

2. Material and methods.

2.1. Reagents, solvents and chlorophyll derivate standards.

2-propanol, Methanol, ethyl acetate, acetonitrile (ACN) and acetone (HPLC grade purity) were supplied by J.T.Baker (Deventer, The Netherlands). The chromatographic solvents were ACN, methanol (LC–MS grade), also from J.T.Baker, and water was purified in a Milli-Q system from Millipore (Bedford, MA, USA). Sodium chloride and ammonium acetate were provided by Fluka (Buchs SG, Switzerland).

Chlorophyll a, chlorophyll b and pheophorbide a were purchased from Sigma-Aldrich. All stock standard solutions were prepared in acetone and stored at –80 ºC. Chlorophyll derivative concentrations were determined spectrophotometrically using a UV2 UV-vis spectrometer (ATI Unicam, Cambridge, UK)[21]. The values of $A_{1\%\ 1\ \mathrm{cm}}$, described in the bibliography, were used to quantify each chlorophyll derivate stock solution. Working solutions were prepared from stock solutions by sampling an aliquot and diluting it with the injection solvent (ACN/acetone (66:33, v/v)). Chlorophyllin sodium copper salt was purchased from Sigma-Aldrich

2.2. Samples.

Commercial luxury teas Needle Silver "white tea", Sencha "green tea", Matcha "green tea", Kenya "black tea" and Pur-eh "red tea" were supplied by Tea Shop in Lleida, Spain. The commercial common teas were purchased from local supermarkets in Lleida, Spain.

Kiwi jams, plum jams, green pepper jam, zucchini puree, green vegetable puree and a melon jam 2 were purchased from local supermarkets in Lleida. Leek puree, asparagus paté, tomato jam, kiwi juice and melon jam 1 were purchased from local supermarkets in Andorra la Vella, Andorra. Pistachio liqueur was purchased from local market in Sicily, Italy.

2.3. Extraction procedure.

In tea samples, pigments were extracted following the procedure described by Suzuki et al.[11] Ten mg of tea were extracted with 2 mL of 80 % of cold aqueous acetone filtered and directly injected.

To extract chlorophyll derivatives from various food commodities such as juices, purees and jellies, a procedure was performed based on proposed procedure by Scotter et al.[18]. One to 4 g of sample was weighted into a 15 mL centrifuge tube were continuously shaken at 5000 rpm (Multi Reax; Heidolph, Schwabach, Germany) for 10 min in a dark room with 4 mL of acetone. Then, 6 mL of ethyl acetate was added and the tube was shaken again as before. Next, 2.5 mL of a solution of NaCl (10%, w/v) was added and the mixture was shaken for 15 min and cooled at 4 °C. The mixture was centrifuged at 2700 × g for 3 min at 4 °C, and the organic layer was recovered in a 10-mL glass tube and dried under nitrogen stream at room temperature. The residue was stored at −80 °C under nitrogen until UPLC analysis. The dry residue was dissolved in the injection solvent immediately prior to analysis.

2.4. Preparation of Standard Solutions.

The chlorophyllides extract was obtained from spinach leaves lyophilised at -50ºC, 1.1 P for 24 h in a Cryodos-50 lyophilizer (Telstar, Terrassa, Spain). The chlorophyllides extraction was performed by the action of the endogenous enzyme chlorophyllase on chlorophylls, by incubating 5 g of dehydrated spinach with 100 mL acetone/0.2 M Tris–HCl buffer (pH 8.0, 1:1 v:v) for 2 h in the dark at 40ºC. The chlorophyllide fraction – the purified mixture of chlorophyllide forms (e.g.Chlid a and Chlid b) – was isolated from the extract by column chromatography with silica gel as the adsorbent (silica gel 60, Merck, 0.063-0.200 mm) and the n-hexane/acetone mixture as the eluent. The n-hexane/acetone ratio was changed from the initial 1:0 to final 1:10, to allow an easier elution of the polar fractions. The chlorophyllide fraction was eluted at the eluent composition 1:3.3 (n-hexane/acetone, respectively) [22]. The final chlorophyllide extract was a mixture of pigments containing large amounts of various chlorophyllide forms, as well as their derivatives, in smaller amounts.

A pheophytins standard solution was prepared by acidification of the chlorophylls standard solution (chlorophyll a and b). Hydrochloric acid (1N) was added dropwise to 100 mL of the chlorophyll standard solution in acetone, and the solution was shaken after each addition, complete conversion was considered once the colour turned from green to olive brown [23].

Pyrochloropylls and pyropheophytins solutions, 10 mL of standard solution chlorophylls and 10 mL of pheophytins solution (in the case of pheophytin solution previous neutralisation with 0.1 M NaOH) was extracted with diethyl ether and the ether extract evaporated to dryness. The residue was dissolved in 20 mL of pyridine and heated under reflux in an oil bath at 100ºC during 2h. Pyridine was removed in a rotary evaporator, and 10 mL of an acetone-water 80:20 mixture was added [7].

2.5. Ultra performance liquid chromatography.

Chromatographic analysis was performed on a Waters ACQUITY UPLC™ system (Waters, Milford, MA, USA) consisting of ACQUITY UPLC™ binary solvent manager and ACQUITY UPLC™ sample manager, coupled to a photodiode array (PDA) 2996 detector. Compounds were separated with an ACQUITY UPLC™ HSS T3 column (1.8 μm; 2.1 mm × 100 mm) (Waters, Manchester, UK) and a gradient system with the mobile phase consisting of solvent A MeOH/iPrOH/ACN (10/15/75, v/v/v), and solvent B, MeOH/ACN/H_2O ammonium acetate 10 mM/ (25/25/50, v/v/v). The linear gradient was as follows: 0–0.68 min, 0% B, 0.6 mL/min (isocratic); 0.68–4.90 min, 50% B 0.6 mL/min (linear gradient); 4.9–9min, 100% B, 0.6 to 1 mL/min (linear gradient); 9–13.7 min, 100% B, 1 mL/min (isocratic); 13.7-14 min, 0% B, 0.6 mL/min (linear gradient); and 13.7–15 min, 0% B, 0.6 mL/min (isocratic). Weak and strong needle solvents were ACN–MeOH (7.5:2.5, v/v) and 2-propanol, respectively. The injection volume was 5 μL and the column was kept at 45 °C while the temperature in the sample manager was maintained at 25 °C.

Instrument control, data acquisition and processing were carried out using MassLynx™ software (version 4.1; Waters, USA).

2.6. Mass spectrometry.

MS analysis was carried out on a Waters ACQUITY TQD tandem quadrupole mass spectrometer (Waters, UK). When instrument was operated using an atmospheric pressure chemical ionisation (APCI) source in positive ion mode, the parameters were as follows: corona voltage 4.0 kV; extractor voltage 3 V; source temperature 150 °C; probe temperature 450 °C; desolvation temperature 150 °C; cone gas (nitrogen) flow 10 L/h; and desolvation gas (nitrogen) flow 150 L/h. When instrument was operated using an electrospray ionisation (ESI) source in positive ion mode, the parameters were as follows: Cone voltage 55 V; capillary voltage 5kV; extractor voltage 3 V; source temperature 130 °C; desolvation temperature 250 °C; cone gas (nitrogen) flow 1 L/h; and desolvation gas (nitrogen) flow 650 L/h. In both cases collision-induced dissociation was achieved using argon as the collision gas at a flow rate of 0.15 mL/min in the collision cell. Data acquisition was performed using MassLynx 4.1 software (Waters, USA).

3. Results and discussion.

3.1. UPLC optimisation.

Here, we tested two columns, namely the ACQUITY UPLC BEH C18 and the ACQUITY UPLC HSS T3, both C18 reversed-phases. The strategy to achieve the best separation was based on optimise in first instance the separation of dephytyled chlorophyll derivatives, the more polar ones, and after, the separation of non-polar chlorophyll derivatives containing phytyl chain.

Regarding column chemistry optimisation, the HSS T3 column retains more the polar chlorophyll derivatives (chlorophyllides and chlorins) and enhances their resolution than the BEH C18 column. However, HSS T3 also retained more nonpolar derivatives, increasing the total analysis time which causes worsening of limit of detection (LOD) and quantification (LOQ) because peak broadening. Since exists different strategies to reduce analysis time in UPLC systems, here we chose HSS T3 column due to its better performance to solve the more complicated separation of the polar compounds.

To optimise the separation of polar dephytyled chlorophyll derivatives, which are acidic pigments ($2<pK_a<4$) [24], ion-pairing techniques must be used to achieve an optimal chromatographic resolution [12, 14, 15]. Here, we used 10 mM of ammonium acetate on column, which is a fully compatible buffer concentration to operate with MS [25]. Simultaneously, optimising the slope of the first part of the gradient and initial water percentage (50%), allowed us to obtain a chromatographic resolution as the best described to date for dephytylated chlorophyll derivatives and chlorin type compounds from E-141 (ii) colorant [12, 15].

To optimise separation of non-polar phytyled chlorophyll derivatives and decrease total time analysis and improve peak shape, a ramp increasing the flow rate up to 1 mL/min was incorporated and column temperature was set at 45 ºC to reduce backpressure. In addition, a 15% of isopropanol (iPrOH) was added to the organic mobile phase (MeOH/iPrOH/MeCN) to increase its eluotropic strength and also reduce time analysis. Different ratios of organic solvent composition were tested, maintaining iPrOH constant at 15%. To do this, black tea which is rich in pheophytins and other non-polar chlorophyll derivatives was used. Figure 1 shows the chromatograms obtained at different % of MeOH (0, 10 and 35 %) in which is observed that a complete separation could not be obtained under any of the conditions tested. As can be seen, some analytes were more affected than others by MeOH composition, as for example hydroxypheophytins (peaks 31, 32, 39 and 40) which showed more retention time variation than pheophytins and pyropheophytins (peaks 34, 35, 42, 43 and 47). This fact, can be explained by the presence of hydroxyl group recognition in hydroxypheophytins by hydrogen-bonding donor and acceptor mechanism. Thus, an organic phase composition of 10/15/75

MeOH/iPrOH/ACN (v,v,v,) was chosen as optimum to perform the analysis of chlorophyll derivatives.

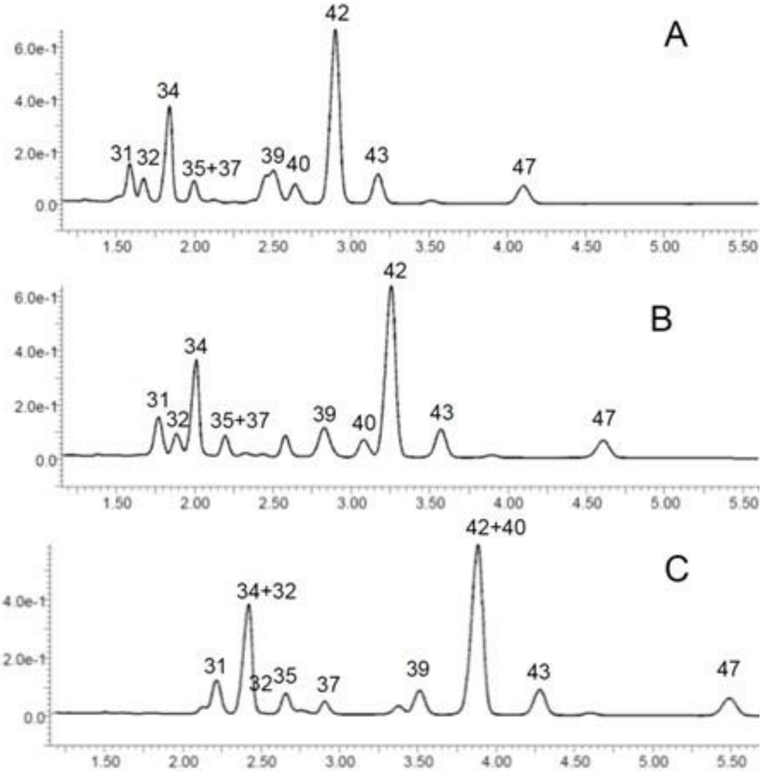


Figure 1. Chromatographic separations of pheophytins and other chlorophyll derivatives of a commercial common black tea at different compositions of solvent B; A: 35/15/50, B: 10/15/65 and C: 0/15/85 of MeOH/iPrOH/MeCN (v/v/v). Measured at 400, 410, 430 and 460 nm and numbered according to Table 1 using HSS T3 column.

The final optimised gradient allowed the separation of mainly chlorophyllide, pheophorbide, chlorophyll and pheophytin derivatives present in processed vegetables, fruit, and teas. This study is the first to describe the separation of all these chlorophyll derivatives by UPLC and greatly improves the previously reported HPLC methods [11, 14, 15, 26]. This improvement is due to greater peak efficiencies obtained by UPLC and HSS T3 column for dephytylated compounds and chlorins. In addition the separation of main chlorophyll and pheophytin chlorophyll derivatives was shortened to 11.2 min, being the fastest separation reported until now.

3.2. Identification.

Identification of many chlorophyll derivatives with the corresponding standard was limited because these standards are not commercially available. Therefore various tools to identify the maximum number of chlorophyll derivatives have been applied. Some chlorophyll derivatives were prepared as described in Material and Methods section. The published data about chromatographic behaviour on C18, about main chlorophyll derivatives in studied matrices and UV-vis absorption spectra (namely, the position of the absorption maxima, i.e. the Soret (S) and Q-bands (Q) and S/Q absorbance ratios) were used to identify. Furthermore, using the molecular ion and fragmentation patterns of MS/MS, we also confirmed the structures of the chlorophyll derivatives. For more polar compounds, was used ESI in positive ionisation. In contrast, in the apolar compounds was used APCI in positive ionisation (Table1).

Table 1. Chromatography, UV-vis and MS parameters of chlorophyll and derivatives found in the samples.

peak	r.t min	Chlorophyll derivatives	Ionisation (mode)	λmax (nm)	S/Q ratio	Precursor ion [M+H]+ (m/z)	MS/MS
1	0.71	Cu-chlorin e6	ESI (+)	409, 640	2.53	657, 626, 614	-
2	0.92	Cu-chlorin p6	ESI (+)	408, 639	2.63	643, 627, 600, 582	-
3	1.81	Chlorophyllide b	ESI (+)	460, 600, 648	2.90	628	-
4	2.78	Chlorophyllide b'	ESI (+)	460, 600, 648	2.90	628	-
5	2.82	Cu-ochlorin e4	ESI (+)	408, 635	3.26	613	-
6	2.82	Cu-isochlorin e4	ESI (+)	408, 635	3.41	569	-
7	3.07	Chlorophyllide a	ESI (+)	430, 575, 617, 664	1.23	615	-
8	3.65	Pheophorbide b	ESI (+)	439, 5,31, 599, 653	4.20	607	-
9	4.1	Chlorophyllide a'	ESI (+)	430, 575, 617, 664	1.23	615	-
10	4.2	Pheophorbide b'	ESI (+)	439, 5,31, 599, 653	4.20	607	-
11	4.98	Cu-porphyrin of chlorin e4	APCI (+)	405	3.33	567	-
12	5.35	Pheophorbide a	ESI (+)	410, 507, 538, 609, 665	2.66	593	533 (10), 505 (50)
13	5.62	Pyropheophorbide a	ESI (+)	410, 507, 538, 609, 665	2.15	535	-

Table 1. Cont.

peak	**r.t min**	**Chlorophyll derivatives**	**Ionisation (mode)**	**λmax (nm)**	**S/Q ratio**	**Precursor ion [M+H]+ (m/z)**	**MS/MS**
14	5.65	Cu-pheophorbide a	ESI (+)	400, 423, 650	1.11	654	-
15	5.85	Pheophorbide a'	ESI (+)	410, 507, 538, 609, 665	2.66	593	533 (10), 505 (50)
16	5.88	Cu-porphyrin of isochlorin e4	APCI (+)		-	611	-
17	6.06	Cu-pyropheophorbide a	ESI (+)	400, 423, 650	-	596	-
18	6.12	Cu-pheophorbide a'	APCI (+)		-	625	-
19	6.22	Cu-porphyrin	APCI (+)		-	583	-
20	7.14	Et-pyropheophorbide b	APCI (+)	436, 529, 560, 600, 658	-	576	-
21	7.76	Et-pyropheophorbide a	APCI (+)	410, 507, 538, 608, 665	-	562	-
22	8.04	Cu-Et-pyropheophorbide a	APCI (+)	400, 425, 651	-	623	-
23	8.74	13^2OH-Chlorophyll b	APCI (+)	464, 599, 648	2.58	924	-
24	8.78	13^2OH-Chlorophyll b'	APCI (+)	464, 599, 648	2.58	924	-
25	8.94	Chlorophyll b	APCI (+)	464, 599, 648	2.86	908	629 (30), 569 (40)
26	9.01	15^1OH-Lactone-pheophytin b	APCI (+)	426, 524, 560, 600, 653	6.80	918	639 (30), 552 (40)
27	9.15	Chlorophyll b'	APCI (+)	464, 599, 648	2.86	908	629 (30), 569 (40)
28	9.21	13^2OH-Chlorophyll a	APCI (+)	464, 599, 648	2.58	910	
29	9.31	13^2OH-Chlorophyll a'	APCI (+)	464, 599, 648	2.58	910	
30	9.39	Chlorophyll a	APCI (+)	431, 617, 663	1.29	894	555 (40), 615 (30)
31	9.5	13^2OH-Pheophytin b	APCI (+)	434, 524, 560, 598, 654	5.29	902	605 (30), 623 (20)
32	9.55	13^2OH-Pheophytin b'	APCI (+)	434, 524, 560, 598, 654	5.29	902	605 (30), 623 (20)
33	9.6	Chlorophyll a'	APCI (+)	431, 617, 663	1.29	894	555 (40), 615 (30)
34	9.67	Pheophytin b	APCI (+)	436, 522, 560, 596, 653	5.56	886	607 (40), 547 (20)

Table 1. Cont.

peak	r.t min	Chlorophyll derivatives	Ionisation (mode)	λmax (nm)	S/Q ratio	Precursor ion [M+H]+ (m/z)	MS/MS
35	9.8	Pheophytin b'	APCI (+)	436, 522, 560, 596, 653	5.56	886	607 (40), 547 (20)
36	9.81	Cu-pheophytin b	APCI (+)	442, 643	2.67	899	-
37	9.82	15^1OH-Lactone-pheophytin a	APCI (+)	400, 500, 530, 668	3.68	904	625 (30), 538 (40)
38	9.92	Cu-pheophytin b'	APCI (+)	442, 643	2.67	899	-
39	10.02	13^2OH-Pheophytin a	APCI (+)	408, 504, 532, 608, 665	2.62	888	593 (30), 610 (30)
40	10.13	13^2OH-Pheophytin a'	APCI (+)	408, 504, 532, 608, 665	2.62	888	593 (30), 610 (30)
41	10.27	Pyropheophytin b	APCI (+)	436, 529, 560, 600, 658	5.23	828	550 (30), 522 (40)
42	10.28	Pheophytin a	APCI (+)	409, 506, 536, 608, 665	3.00	872	593 (40), 533 (40)
43	10.47	Pheophytin a'	APCI (+)	409, 506, 536, 608, 665	3.00	872	593 (40), 533 (40)
44	10.6	Cu-pyropheophytin b	APCI (+)	443, 643	2.14	889	-
45	10.73	Cu-pheophytin a	APCI (+)	400, 423, 650	1.02	932	-
46	11.01	Cu-pheophytin a'	APCI (+)	400, 423, 650	1.02	932	-
47	11.22	Pyropheophytin a	APCI (+)	410, 507, 538, 608, 665	2.87	814	535 (40)
48	12.21	Cu-pyropheophytin a	APCI (+)	400, 425, 651	1.08	875	-

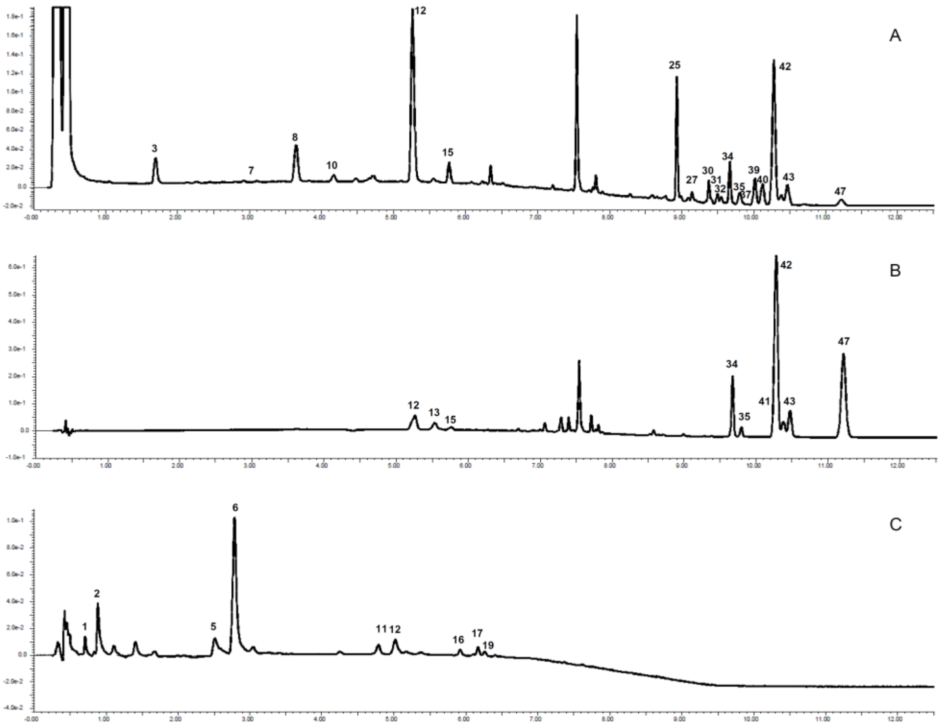


Figure 2. (A) "Kenya" Black tea; (B) commercial green pepper jam; and (C) chlorophyllin sodium copper salt commercial grade. All samples were analyzed at 400, 410, 430 and 460 nm. Compounds numbered according to Table 1.

Several publications have noticed four chromatographic clusters of peaks on columns with C18 stationary phases, corresponding to chlorophyllides, pheophorbides, chlorophylls and pheophytins, in increased retention order. Each cluster includes the peak of the main pigment (chlorophyll / chlorophyllide / pheophytin / pheophorbide, either a or b), the peak of its epimer forms and the peak of the pyro forms. Figure. 2 (A and B) shows the identification of several chlorophyll and their derivatives in a Kenya Black tea and green pepper jam using the criteria mentioned above. Peaks 25, 30 and 12 were conclusively identified as chlorophyll b, chlorophyll a and pheophorbide a respectively, by comparison with commercial standard. Peaks 3, 7, 8, 34, 42, 41 and 47 were assigned as chlorophyllide b and a, pheophorbide b, pheophytin b and a, and pyropheophytin b and a, respectively; according to retention behaviour, absorption spectra and confirmed with standard solution from spinach extract degraded (Material and Methods section). In addition, mass spectrometry confirmed chlorophyll species phytylated through $[M+H]^+$ and their product ions, using APCI (+) ionisation. Into the chlorophyll species dephytylated pheophorbides were confirmed through $[M+H]^+$ ions and chlorophyllides were confirmed through $[M]^+$ and their product ions, using ESI (+) ionisation [22]. The corresponding epimers of the main chlorophyll derivatives, a' from a and b' from b, have identical MS/MS fragmentation patterns. However, the epimers a' and b' applying the same energy collision show a mass spectrum where the abundance of dephytylated ion gives greater relative abundance against ion from loss of 60 m/z ($HCOO+CH_3$) than a and b epimers, as is related in earlier publications [27]. This can be noted in the different collision energy established to precursor ions in the performance of MRM method (Table 1). The 13^2OH-chlorophylls and 13^2OH-pheophytins were identified with $[M+H]^+$ and their product ions which presented a characteristic loss of 17 amu $[M+H-OH]^+$. 15^1OH-Lactone-pheophytin a (peak 37) and b (peak 26), were also identified through his characteristic UV-vis spectra maximums at 400 and 426 nm and 668 and 653 nm, respectively, and confirmed through their pseudomolecular ions. In addition is described for first time the 15^1OH-Lactone-pheophytin fragments, Figure 3. 15^1OH-Lactone-pheophytin a shows the corresponding fragmentation pattern that leads to the loss of hydroxyl $[M+H-OH]^+$ at m/z 886, the loss of phytyl chain $[M+H-C_{20}H_{38}]+$ at m/z 625 and loss of phytyl and ester chain $[M+H-CH_3COO\text{-phytyl}]$ at 537 m/z. Moreover the ions at m/z 580 and 538.3 were the selective fragments useful to identify this chlorophyll derivative.

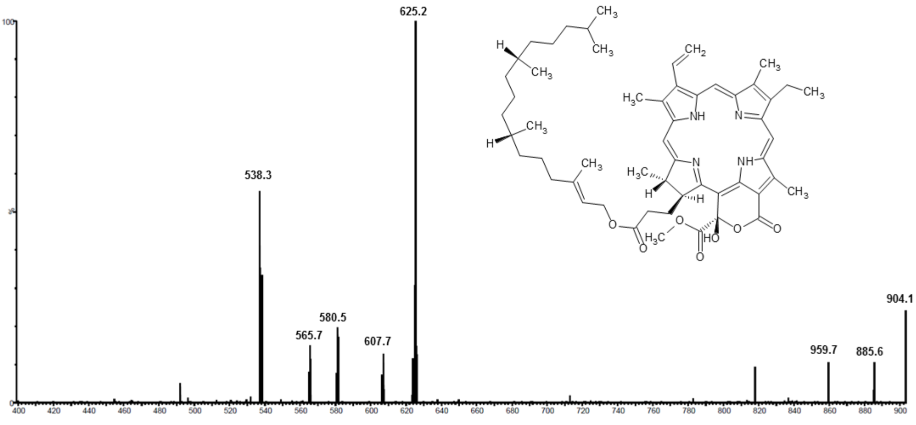


Figure 3. MS/MS daughter ions of 15^1OH-Lactone-pheophytin.

To identify E-141 copper chlorophylls and chlorophyllins were used combination of UV-vis spectroscopy and mass spectrometry. E-141 (i) colorant is a mixture of copper derivatives soluble in oil, mainly copper pheophytin a and pyropheophytin a as it has been described in other publications [28, 29]. The copper ion incorporation in a chlorophyll derivative induces a bathochromic displacement of the Soret band (between 3 and 15 nm depending the pigment) and a hypsochromic displacement of Q band (between 12 and 30 nm depending the pigment). The copper ion in a chlorophyll derivatives also alters its polarity, increasing his retention time in the chromatographic column respect their analogue, for example, retention time of Cu-pheophytin a was increased in 0.45 min from pheophytin a (10.28 min). Identification was also assisted by mass spectrometry, because coppered compounds show a distinctive isotopic splitting due to copper and carbon isotopes [17]. However, the fragmentation patterns from molecular ion were very similar than described for their uncoppered analogues. In the studied samples were tentatively identified Cu-pheophytin a and b (peaks 45 and 34),Cu-pyropheophytin a and b (peaks 48 and 44), Cu-pheophorbide a (peak 14), Cu-pyropheophorbide a (peak 17) and Cu-Et-pyrophophorbide (peak 22).

The colorant sodium copper chlorophyllin (E-141 (ii)) is a commercial food additive grade composed of a mixture of compounds soluble in water derived from natural chlorophyll by saponification and copper insertion, their chromatographic profile is showed in Figure 4. The profiles of peaks in sodium copper chlorophyllin standard and in samples to which it has been added as additive were similar. This profiles exhibited spectroscopic characteristics typical of derivatives with Cu-chlorin type structures which have been previously reported in the literature [1, 17, 18, 30]. Mass spectroscopic data was gathered using APCI and ESI in positive mode, and in general were obtained better signals in ESI for the more polar compounds of the chlorin mixture (Table1). Copper isochlorin e4 (peak 6) was the main compound, their presence was supported considering that the maximum of the UV-vis spectrum at 408 nm and 635 nm with a shoulder at 400 nm, in addittion it was confirmed by molecular ion at 613 m/z. Other minor chlorin type compounds such as copper chlorin e4 (peak 5), chlorin e6 (peak 1), chlorin p6 (peak 2) were also identified based on spectroscopic data and chromatographic behaviour. Phorphyrins were easily identified by their absorption spectra, and lack the intense of Q-band maximum at around 630 nm. Cu-porphyrin of chlorin e4, Cu-porphyrin of isochlorin e4, and Cu phorphyrin were confirmed by mass spectrometry through their molecular ion which is two units low than their chlorin analogue due to oxidation of the chlorins to porphyrins.

3.3. Application of method developed.

The method developed was applied to analyse samples that have been suffered intense transformation during its manufacturing. Thereby, we selected various tea types to assess the capacity of the analytical method to distinguish between them and their level of processing. In our study we decided to quantitate chlorophylls and their coloured derivatives over different types and varieties of teas: In addition we attempted a comparison of chlorophyll derivatives between supermarket own-brand tea bags and offered teas by specialised shops so-called luxury rivals, which *a priori* offers high quality and treat of product with respect and meticulousness along all the commercial chain. Other difference in "luxury" teas is the control and specificity of the variety, while commercial common teas generally exhibit mixtures without specifying their varieties. Several publications have described the profile of chlorophyll derivatives in tea [11, 31, 32], but an accurate description of the amount of chlorophyll and their derivatives to be used as quality indicator has not been achieved so far. Focusing on chlorophyll content, it was higher in "luxury" teas than their supermarket own-brand counterparts. However, in green and white types the differences were higher than found in black teas. In Pur-eh tea were not found chlorophyll compounds, this fact may relate to the process of degradation of chlorophylls during the process of double fermentation carried out in the manufacturing process of these teas. The highest content of chlorophyll was showed by Matcha variety with 3579.3 ± 64.9 μg/g, distributed between chlorophyll a and b 1248.8 ± 26.4 and 1310.2 ± 17.7 μg/g, respectively, these data are agree with the fact that keeping the characteristic green color of this powdered green tea. Matcha is followed even distantly by Silver needle, Sencha and Kenya varieties with values around 1600 μg/g. Among the supermarket own-brand black tea showed values of chlorophyll of 1310.0 ± 31.2 μg/g, while white and green types showed much lower values of 885.7 ± 299.9 and 349.0 ± 24.4 μg/g, respectively.

The conversion rate of chlorophyll to chlorophyll derivatives such as pheophytin and pheophorbide is a very useful indicator of degree of discoloration and the quality tea. Figure 4 shows the different chromatographic profile in pheophytin and chlorophyll zone of two luxury green teas Sencha and Matcha and a commercial common green tea. In terms of relationship between chlorophyll derivatives and chlorophylls, supermarket own-brand teas showed higher values than their counterparts from specialty stores. This fact may be related to several factors followed by these speciality stores such as: modern blending and packaging system to ensure the freshness and quality of product at every step of the supply chain or ensuring the necessary cool and dry environment during storage. However, pheophytins and pheophorbides as the degradation intermediates are also important to observe the degree of degradation of teas before total deterioration of chlorophyll and chlorophyll derivatives.

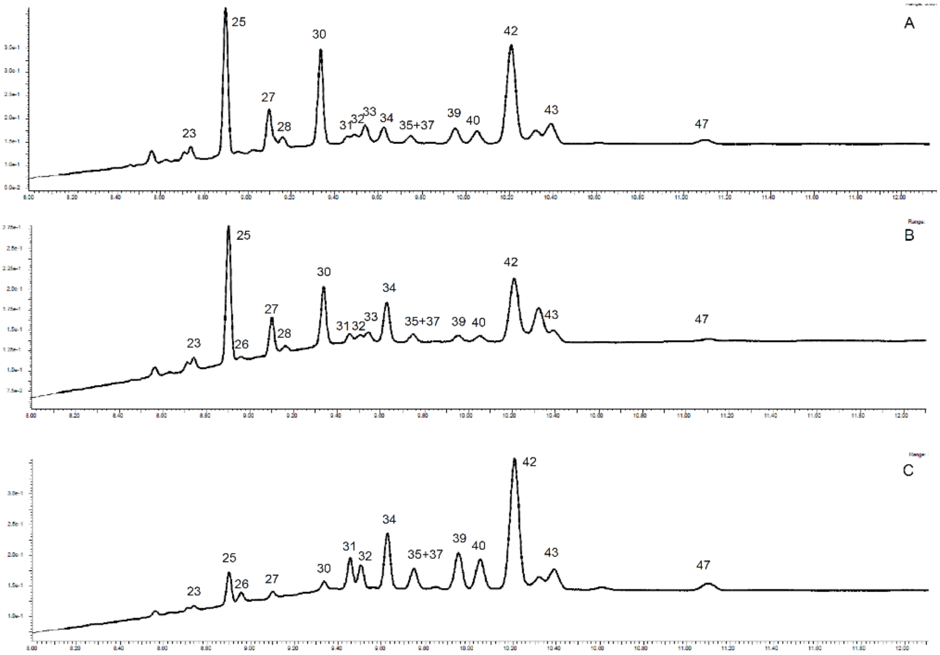


Figure 4. Chlorophyll and chlorophyll derivatives profile over the range 8.00–12.5 min at 400, 410, 430 and 460 nm for Matcha (A), Sencha (B) and commercial common (C) black teas. Numbered according Table 1.

We can see how all types and varieties of teas contain these compounds as well as in all the amount of a compound form is higher than b compound form due to his major instability (Table 2) [32]. Sencha exhibited the higher values of pheophorbide and pheophytin, with 3226.8 ± 320.2 and 353.3 ± 43.7 μg/g of pheophytin a and b, and with 1255.4 ± 123.7 and 225.8 ± 35.3 μg/g of pheophorbide a and b. High quantities of pheophytin a were also found in Matcha 2524.3 ± 40.97 μg/g, but were not found pheophorbides. In black teas and commercial common white and green teas also exhibited high quantities of pheophorbides and pheophytins, however Silver Needle presented the lowest values 500.8 ± 46.0 μg/g. On the other hand in Pur-eh tea the values were high but in this case the values between a and b forms were more similar in pheophytin, problably due to their high degradation in pheophorbides, in these case a form was higher than b. In content of pyropheophytin a, Pur-eh was also the tea with the highest content both in the commercial common tea, 246.7 ± 27.5 µg/g, as its luxury counterpart, 326.8 ± 26.3 µg/g. Agreeing as described in previous publications, it appears that the process of pan-firing carried out in these teas degrades the chlorophylls much more that no-oxidation processes which that are specific to those of the black tea [11, 33].

Table 2. Chlorophyll derivative content (μg/g) of various types of tea.

Chlorophyll derivative	Commercial white tea	White tea "Silver needle"	Commercial green tea	Green tea "Sencha"	Green tea "Matcha"
Chlorophyllide b				123.1 ± 15.7	
Chlorophyllide b'		95 ± 9.1		129.1 ± 13.3	
Chlorophyllide a	76.6 ± 2.1	135.1 ± 8.7		135.3 ± 13	
Chlorophyllide a'		86.8 ± 6.2			
Pheophorbide b	168.5 ± 10.5	72.4 ± 7.3	90.1 ± 6.8	225.8 ± 35.3	
Pheophorbide b'	53.6 ± 1	53.6 ± 0.9	52 ± 1.8		
Pheophorbide a	563 ± 40	312.7 ± 40.7	219 ± 26.2	1255.4 ± 123.7	
Pheophorbide a'	118.9 ± 7	72.9 ± 4.7	68.7 ± 2.5	167.7 ± 16.3	
13^2OH-Chlorophyll b	81.4 ± 12.9	41.8 ± 3.3	61.8 ± 6.7	150 ± 12.7	226.3 ± 8.3
Chlorophyll b	448.6 ± 236.4	632.4 ± 82.7	159.5 ± 11	758.2 ± 78.1	1310.2 ± 17.7
15^1OH-Lactone-pheophytin b	51.4 ± 2.4		70.9 ± 8.1	41.5 ± 4.1	
Chlorophyll b'	54.7 ± 5.9	72.5 ± 8.1	55.3 ± 4.1	227.4 ± 26.3	409.7 ± 6.6
13^2OH-Chlorophyll a					110.6 ± 4.8
Chlorophyll a	301 ± 44.7	931.4 ± 91.2	72.4 ± 2.6	415.8 ± 37	1248.8 ± 26.4
13^2OH-Pheophytin b	118 ± 6.9		166.9 ± 11.1	77.5 ± 10.5	97.8 ± 6.3
13^2OH-Pheophytin b'	93.8 ± 6.8	36.1 ± 2.3	140.6 ± 5.6	77.5 ± 10.1	159.3 ± 2
Chlorophyll a'				90.4 ± 4.9	273.8 ± 1.2
Pheophytin b	321.4 ± 19.9	61.1 ± 0.8	368.2 ± 18	353.3 ± 43.7	109.3 ± 4.4
Pheophytin b'	101.2 ± 3.4		106.5 ± 10.7	89.5 ± 7.2	
15^1OH-Lactone-pheophytin a	144.5 ± 12.5	68.6 ± 6.3	222.5 ± 2.3	156.8 ± 21.8	190 ± 3.7
13^2OH-Pheophytin a	295.7 ± 23.8	83.5 ± 13.2	470.5 ± 27.2	318.4 ± 45	407.6 ± 7
13^2OH-Pheophytin a'	230.4 ± 16.1	68.4 ± 2.7	390.9 ± 23.6	270.4 ± 37.1	366.9 ± 7.7
Pheophytin a	1597.8 ± 56.1	500.8 ± 46	1866.9 ± 121.7	3226.8 ± 320.2	2524.3 ± 41
Pheophytin a'	289.7 ± 34	130.7 ± 7.1	339.9 ± 15.7	573 ± 44.1	490.3 ± 12.7
Pyropheophytin a	235.9 ± 37.6	72.8 ± 6.3	177.4 ± 9.6	163.4 ± 17	241.9 ± 10.8

Table 2. Cont.

Chlorophyll derivative	Commercial black tea	Black tea "Kenya"	Commercial "Pu-erh" Tea	"Pu-erh" Tea
Chlorophyllide b	70.5 ± 2.4	92.6 ± 9.8		
Chlorophyllide b'	85.3 ± 3.3	104.8 ± 6.3		
Chlorophyllide a	81.2 ± 2	136.4 ± 8.1		
Chlorophyllide a'				
Pheophorbide b	193.9 ± 5.6	140.4 ± 11.3	224 ± 1.6	321.3 ± 27.4
Pheophorbide b'			105.5 ± 7.1	219.1 ± 1.8
Pheophorbide a	1016.5 ± 33.8	631.8 ± 52.2	511.6 ± 4.7	881.9 ± 47.9
Pheophorbide a'	149.3 ± 2.3	98.8 ± 9.3	114.6 ± 6.4	294.8 ± 5.3
13^2OH-Chlorophyll b				
Chlorophyll b	705.2 ± 15.6	796.5 ± 51.4	50.6 ± 0.3	
15^1OH-Lactone-pheophytin b			43.1 ± 3.4	114.4 ± 11.5
Chlorophyll b'	63.3 ± 1	59.1 ± 2.7	30.7 ± 2.1	
13^2OH-Chlorophyll a				
Chlorophyll a	541.5 ± 14.6	788.4 ± 51.3		
13^2OH-Pheophytin b	62 ± 2.7	52.5 ± 3.6	76.3 ± 10.3	149.4 ± 14.6
13^2OH-Pheophytin b'	52.6 ± 3.4	52.5 ± 3.6	63.7 ± 9.3	125.4 ± 11.1
Chlorophyll a'				
Pheophytin b	237.1 ± 5.4	112.4 ± 7.1	248.6 ± 15.5	222.1 ± 18
Pheophytin b'	58.4 ± 1.9		68.4 ± 7.1	60 ± 5.7
15^1OH-Lactone-pheophytin a	85.3 ± 0.9	103.2 ± 1.9		
13^2OH-Pheophytin a	169.8 ± 8.2	192.5 ± 7.1	121.4 ± 16.2	251.8 ± 25.2
13^2OH-Pheophytin a'	130.8 ± 2.4	166.6 ± 6.8	100.6 ± 17.1	214.9 ± 24.5
Pheophytin a	1184.9 ± 9.9	758.4 ± 58.7	587.2 ± 10.7	587.6 ± 56
Pheophytin a'	176.5 ± 6.8	136.4 ± 11.2	98.1 ± 0.6	95.9 ± 9.6
Pyropheophytin a	124.2 ± 8.7	98.3 ± 10.9	246.7 ± 27.5	326.8 ± 26.3

For the identification of derivatives of chlorophyll derivatives in tea it had never been used mass spectrometry, in this work we applied this tool to confirm the presence of 13^2OH- and 15^1OH-lactone in chlorophyll derivatives which to date had not described in this matrix. The identification was based on publications of chromatography coupled to mass spectrometry which analysed similar matrix, such as medicinal herbs and aromatic herbs [15, 34, 35]. 13^2OH- and 15^1OH-lactone have been described as intermediary products in thermal degradation mechanism that affect the porphyrin ring, producing colourless compounds in oils [36]. In general they content and ratio a/b were according to profile of pheophytins, however the 13^2OH-pheophytins and chlorophylls were epimerised in ratio 1:1 between a and a'. In the case of 15^1OH-lactone only one peak was found. Finally chlorophyllide a and b were found in some teas, it is important because they are green pigments water soluble, so the infusion are those that give green colour. Finally, we can conclude that the method developed allows us to characterise the different varieties of tea and asses how it processing affects their chlorophyll derivatives profile. Likewise also it allows us the degree of degradation and the large number of compounds formed from the degradation of chlorophylls in tea processing thanks to the excellent sensitivity of the methodology.

The developed methodology has also been applied in other samples obtained from green vegetables, such as purees, jams, juices and other vegetable products (Table 3 and 4). These samples are characterised by a higher processing using various temperature stages such as blanching, canning and retorting. Contrary to happened in any of the tea samples it was detected chlorophylls or chlorophyllides. This can be in accordance with publications describing an almost complete conversion of chlorophyll to pheophytinized derivatives during prolonged heat treatments [6, 37]. In terms of total chlorophyll derivatives green pepper jam was the one that showed the highest amounts, with values of 5018.0 ± 498.9 μg/100g, followed by the puree mixture of green vegetables and green asparagus pate with values of 4430.7 ± 380.6 and 4295.8 ± 115.4 μg/100g, respectively. Leek and zucchini purees contents were close to 3700 μg/100g, while kiwi jams showed an average content of 2100 μg/100g. The other jams chlorophyll derivatives contents ranged between 450 and 1400 μg/100g depending on the product, being the lowest for the one melon jam with 460.6 ± 10.0 μg/100g.

Table 3. Chlorophyll derivatives content (μg/g fresh matter) in processed products from green vegetables.

Chlorophyll derivatives	**Zucchini puree**	**Leek Puree**	**Green vegetables puree**	**Asparagus paté**	**Prune jam 1**	**Green pepper jam**
Pheophorbide a	18.9 ± 0.4	49.0 ± 1.0	59.8 ± 6.6	80.9 ± 1.2	65.8 ± 10.4	188.9 ± 25.7
Pyropheophorbide a	39.2 ± 0.7	65.4 ± 3.1	56.6 ± 5.6	82.6 ± 1.2	44.5 ± 8.4	91.2 ± 8.9
Pheophorbide a’	11.4 ± 0.2	16.2 ± 0.7	16.7 ± 1.1	18.7 ± 0.4	17.1 ± 1.5	35.4 ± 3.5
Pheophytin b	289.6 ± 15.6	310.9 ± 18.6	706.8 ± 53.0	626.0 ± 15.4	155.8 ± 8.1	757.7 ± 99.4
Pheophytin b’	122.1 ± 2.4	124.6 ± 2.7	196.3 ± 11.5	192.5 ± 2.9	96.3 ± 1.6	201.9 ± 11.4
Pyropheophytin b	133.5 ± 1.6	125.2 ± 4.2	123.2 ± 4.2	160.1 ± 3.1	84.9 ± 0.2	180.7 ± 10.7
Pheophytin a	945.8 ± 46.1	920.4 ± 85.5	1668.2 ± 156.5	1562.2 ± 59.4	309.6 ± 19.4	1774.9 ± 177.0
Pheophytin a’	151.7 ± 6.0	165.90 ± 8.5	310.7 ± 21.7	301.6 ± 8.1	129.6 ± 0.3	321.4 ± 24.5
Pyropheophytin a	1847.8 ± 58.3	1541.9 ± 192.6	1292.4 ± 120.4	1271.2 ± 23.5	155.1 ± 4.1	1369.3 ± 135.2
Total chlorophyll derivatives	3890.5 ± 131.3	3649.3 ± 316.9	4430.7 ± 380.6	4295.8 ± 115.4	1155.5 ± 56.4	5018.0 ± 498.9

Table 4. Chlorophyll derivatives content (μg/g fresh matter) in processed products from green vegetables.

Chlorophyll derivatives	**Prune jam**	**Tomato jam**	**Melon jam I**	**Melon jam II**
Cu-chlorin p6	75.1 ± 9.3			
Cu-chlorin e4				
Cu-isochlorin e4	2813.1 ± 28.2			
Pheophorbide a	231.9 ± 3.0			32.4 ± 1.2
Pyropheophorbide a	78.2 ± 0.8			12.8 ± 0.5
Cu-pheophorbide a		26.4 ± 1.5		
Pheophorbide a'	61.9 ± 0.4			10.8 ± 0.9
Cu-porphyrin of isochlorin e4				
Cu-pyropheophorbide a		32.2 ± 0.6	10.5 ± 0.6	
Cu-pheophorbide a'				
Cu-porphyrin				
Et-pyropheophorbide b				
Et-pyropheophorbide a				
Cu-Et-pyropheophorbide a				
Pheophytin b	637.2 ± 5.1	124.0 ± 2.7		151.3 ± 0.7
Pheophytin b'	390.9 ± 1.5	88.3 ± 0.7		64.15 ± 0.3
Cu-pheophytin b		120.5 ± 1.6	58.4 ± 1.7	
Cu-pheophytin b'		99.5 ± 1.2	47.2 ± 0.2	
Pyropheophytin b				
Pheophytin a	328.6 ± 12.7	107.5 ± 1.7		275.7 ± 0.7
Pheophytin a'	129.6 ± 0.8	87.3 ± 0.7		86.2 ± 0.2
Cu-pyropheophytin b		144.6 ± 1.3	66.1 ± 1.1	
Cu-pheophytin a		231.4 ± 8.0	70.0 ± 3.2	
Cu-pheophytin a'		123.8 ± 2.6	52.6 ± 1.3	
Pyropheophytin a	100.9 ± 1.6			53.8 ± 0.8
Cu-pyropheophytin a		208.7 ± 4.9	67.6 ± 2.1	
Total chlorophyll derivatives	2041.9 ± 25.7	1394.2 ± 27.5	460.6 ± 10.0	687.13 ± 5.3

Table 4. Cont..

Chlorophyll derivatives	Kiwi jam I	Kiwi jam II	Kiwi juice	Pistachio liqueur
Cu-chlorin p6			263.1 ± 30.5	
Cu-chlorin e4			82.2 ± 0.2	
Cu-isochlorin e4			751.2 ± 28.2	
Pheophorbide a	54.8 ± 3.0	425.1 ± 30.3	16.0 ± 0.3	
Pyropheophorbide a			16.0 ± 0.3	
Cu-pheophorbide a	48.2 ± 5.3			
Pheophorbide a'	16.1 ± 0.5	59.0 ± 3.3	4.1 ± 0.3	
Cu-porphyrin of isochlorin e4			107.1 ± 1.3	
Cu-pyropheophorbide a	16.1 ± 1.5		7.9 ± 0.4	
Cu-pheophorbide a'			2.9 ± 0.2	
Cu-porphyrin			92.6 ± 2.23	
Et-pyropheophorbide b				70.9 ± 4.3
Et-pyropheophorbide a				187.4 ± 2.9
Cu-Et-pyropheophorbide a				83.0 ± 0.9
Pheophytin b	397.8 ± 27.9	656.2 ± 22.5	77.6 ± 2.4	
Pheophytin b'	139.1 ± 4.9	221.5 ± 3.6	31.1 ± 1.9	
Cu-pheophytin b				
Cu-pheophytin b'				
Pyropheophytin b		86.8 ± 0.8	21.33 ± 0.1	
Pheophytin a	584.2 ± 28.8	654.5 ± 6.4	134.3 ± 6.6	
Pheophytin a'	165.3 ± 6.2	218.1 ± 4.0	42.6 ± 1.1	
Cu-pyropheophytin b				214.5 ± 10.4
Cu-pheophytin a	126.4 ± 2.5			125.3 ± 3.5
Cu-pheophytin a'	98.7 ± 0.9			108.1 ± 4.5
Pyropheophytin a	166.1 ± 1.4	112.1 ± 4.2	30.3 ± 0.5	309.15 ± 20.1
Cu-pyropheophytin a				267.07 ± 15.7
Total chlorophyll derivatives	1812.7 ± 82.9	2433.4 ± 74.4	1680.5 ± 76.2	1365.45 ± 62.3

The distribution of derivatives it depends on the processing, thus the generation of pyropheophytin at been associated with the intensity of the heat treatment [38]. In this type of samples the levels of pyropheophytin were higher than found in tea samples. In leek and zucchini purees pyropheophytin a was determined as the predominant chlorophyll derivatives, with values of 1541.9 ± 192.6 and 1847.8 ±

58.3 μg/100g respectively, being between two and one-half upper than pheophytin a content. Similar behavior in the ratio was exhibited for the compounds pyropheophorbide a and pheophorbide a in the samples, however the contents of pyropheophorbide a were lower with 39.2 ± 0.7 and 65.4 ± 3.1 μg/100g for zucchini and leek puree, respectively. Moreover green vegetable puree, asparagus paté and green pepper jam showed high values of these chlorophyll derivatives, close to 1300 μg/100g for pyropheophytin a and around 1600 μg/100g for pheophytin a, also the pheophorbide derivatives were lower in content, but is necessary highlight the contents of this compounds in green pepper jam with 188.9 ± 25.7 μg/100g of pheophorbide a and 91.2 ± 8.9 μg/100g of pyropheophorbide a. On the other hand, in the case of chlorophyll b derivatives in the samples were found Pheophytin b and b' and pyropheophytin b, in contrast with chlorophyll a derivatives in the b derivatives were not found pheophorbide derivatives and in any sample the levels of pyropheophytin b were higher than pehophytin b. The highest levels of pheophytin were showed by green pepper jam, asparagus paté and green vegetables puree with values around 700 μg/100g. Not too far values for pheophytin b was shown by kiwi jams, note that the levels of pheophytin a and b in this case were similar values, as could be expected from the contents described in chlorophylls a and b in fresh fruit [37, 39].

In only two of the samples, kiwi juice and plum jam 1 is declared among the ingredients the use of color E-141, in both the dye is declared the (ii). Copper chloropphyllin are water soluble and are allowed as food colorant within the EU [40]. Processing of chlorophyll to chlorophyllin results in a mixture of a large number of compounds and the composition differs between suppliers [17]. In the commercial grade colorants the main constituents are Cu chlorin p6, and Cu isochlorin e4. In the samples studied the main component was Cu isochlorin e4 and Cu chlorin p6, with values of 7.51 ± 0.28 ug /100g in kiwi juice and 1.9 ± 0.13 μg/100g in plum jam 1 for the first and 263.07 ± 30.54 and 47.6 ± 2.45for the second . Other compounds presents in E 141 (ii) were identified and quantified in the kiwi juice, such as Cu-porphyrin of isochlorin e4, Cu-pyropheophorbide a, Cu-pheophorbide a' and Cu-porphyrin (Figure 5 (A)). Although not stated in the ingredients, several of the products analysed in the study presented copper pheophytins typical coloring E-141 (i), such as Cu-pheophytins, Cu-pyropheophytins, Cu-pheophorbides and Cu-pyropheophorbide [18, 28]. These compounds have been identified in three of the studied jams arising from melon, kiwi 1 (Figure 5 (B)) and tomato. In melon jam all the compounds were copper chlorophyll derivatives, Cu-pheophytins and pyropheophytins a and b were detected and their concentration ranged between 47.2 to 70.0 μg/100g. Finally it is necessary to highlight the pistachio liqueur, the list of ingredients in this product state in the presence of colorants E-100 curcumin and E131 patent blue V, although once analysed also several copper chlorophyll derivatives were identified and all the chlorophyll derivatives identified were copper derivatives.

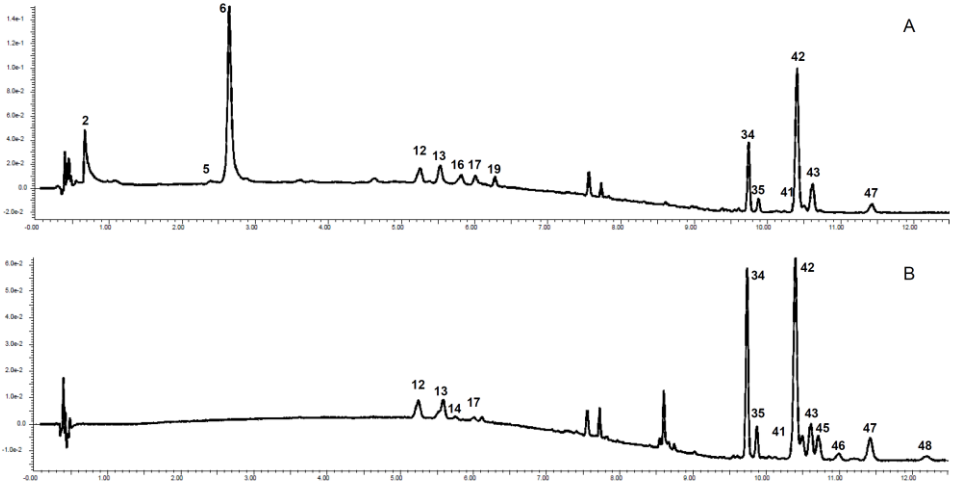


Figure 4. (A) Commercial kiwi juice analysed at 400, 410, 430 and 460 nm; (B) commercial kiwi 1 jam analysed at 400, 410, 430 and 460 nm. Numbered according to Table 1.

4. Conclusions.

Here, we described a UPLC–PDA–MS/MS analytical method that allows the simultaneous determination of chlorophyll a and b and 46 of its derivatives. Separation of chlorophylls and their derivatives was achieved using a ternary gradient in UPLC (MeOH, iPrOH,MeCN and H_2O 10 mM of ammonium acetate) in less than 12 min. The method allowed the proper separation of the chlorophyll derivatives obtained from demetalitation, dephytilation, decarbomethoxylation epimerisation and copperisation for the first time in a UPLC system. This 48 compounds were tentatively identified in teas and processed green vegetable and fruit peach foodstuffs based on chromatographic retention time, UV–vis absorption spectra and MS/MS.

The developed method allowed us to observe the different grade of processing between commercial common teas against luxury teas, since the content of chlorophylls and their derivatives was higher in the teas from specialty shops. In addition, an approach to differentiate among varieties of tea such as, Matcha, Sencha, Silver Needle, Kenya and Pur-Eh showed encouraging results. The method was also applied to study the chromatographic profile in processed fruit and vegetable products, in these samples were determined mainly pehophytin, pheophorbides and pyro derivatives thereof. However, in several products were also detected chlorophyll derivatives food colourants such as: Cu-chlorophyllins, Cu-pheophytins, Cu-pyropheophytins, Cu-pheophorbides and Cu-pyropheophorbides.

References.

[1] S. Chernomorsky, A. Segelman, R.D. Poretz, Teratogenesis, carcinogenesis, and mutagenesis, 19 (1999) 313.

[2] J.Y.-W. Chan, P.M.-K. Tang, P.-M. Hon, S.W.-N. Au, S.K.-W. Tsui, M.M.-Y. Waye, S.-K. Kong, T.C.-W. Mak, K.-P. Fung, Planta Medica., 72 (2006) 28.

[3] K. Hiqashi-Okaj, S. Otani, Y. Okai, Cancer Letters, 140 (1999) 21.

[4] T. Tumolo, U.M. Lanfer-Marquez, Food Research International, 46 (2012) 451.

[5] S. Gaur, U. Shivhare, J. Ahmed, Stewart Postharvest Review, 2 (2006) 1.

[6] F. Canjura, R. Nunes, S., Journal of Food Science, 56 (1991) 1639.

[7] S.J. Schwartz, S.L. Woo, J.H. Von Elbe, Journal of Agricultural and Food Chemistry, 29 (1981) 533.

[8] M.I. Minguez-Mosquera, B. Gandul-Rojas, J. Minguez-Mosquera, Journal of Agricultural and Food Chemistry, 42 (1994) 1089.

[9] J.W. Heaton, A.G. Marangoni, Trends in Food Science & Technology, 7 (1996) 8.

[10] E. Psomiadou, M. Tsimidou, Journal of Agricultural and Food Chemistry, 50 (2002) 722.

[11] Y. Suzuki, Y. Shioi, Journal of Agricultural and Food Chemistry, 51 (2003) 5307.

[12] R. Aparicio-Ruiz, K.M. Riedl, S.J. Schwartz, Journal of Agricultural and Food Chemistry, 59 (2011) 11100.

[13] M. Suman, R. De Maria, D. Catellani, Journal of the Science of Food and Agriculture, 88 (2008) 471.

[14] L. Almela, J.A. Fernández-López, M.a.J. Roca, Journal of Chromatography A, 870 (2000) 483.

[15] J.-L. Lafeuille, S. Lefèvre, J. Lebuhotel, Journal of Agricultural and Food Chemistry, 62 (2014) 1926.

[16] N. Ishida, Journal of Chromatography A, 1218 (2011) 5810.

[17] A. Mortensen, A. Geppel, Innovative Food Science and Emerging Technologies, 8 (2007) 419.

[18] M.J. Scotter, L. Castle, D. Roberts, Food Additives and Contaminants, 22 (2005) 1163.

[19] A. Delpino-Rius, J. Eras, A. Marsol-Vall, F. Vilaró, M. Balcells, R. Canela-Garayoa, Journal of Chromatography A, 1331 (2014) 90.

[20] W. Fu, M. Magnúsdóttir, S. Brynjólfson, B.Ø. Palsson, G. Paglia, Analytical and Bioanalytical Chemistry, 404 (2012) 3145.

[21] S. Jeffrey, R. Mantoura, Development of pigment methods for oceanography: SCOR-supported working groups and objectives, 1997.

[22] S.M. Milenković, J.B. Zvezdanović, T.D. Anđelković, D.Z. Marković, Advanced technologies, 1 (2012) 16.

[23] F. Pennington, H. Strain, W. Svec, J. Katz, Journal of the American Chemical Society., 86 (1964) 1418.

[24] A.P. Gerola, T.M. Tsubone, A. Santana, H.P. de Oliveira, N. Hioka, W. Caetano, The Journal of Physical Chemistry B, 115 (2011) 7364.

[25] L. Feltl, V. Pacakova, K. Stulik, K. Volka, Curr. Analytical Chemistry, 1 (2005) 93.

[26] C.H. Loh, B.S. Inbaraj, M.H. Liu, B.H. Chen, Journal of Agricultural and Food Chemistry, 60 (2012) 6108.

[27] A. Gauthier-Jaques, K. Bortlik, J. Hau, L. Fay, Journal of Agricultural and Food Chemistry, 49 (2001) 1117.

[28] A. Pérez-Gálvez, J.J. Ríos, M. Roca, Food Control, 51 (2015) 240.

[29] M. Roca, L. Gallardo-Guerrero, M.a.I. Mínguez-Mosquera, B. Gandul Rojas, Journal of Agricultural and Food Chemistry, 58 (2009) 51.

[30] B. Gandul-Rojas, M. Roca, L. Gallardo-Guerrero, LWT - Food Science and Technology, 46 (2012) 311.

[31] A. Loranty, E. Rembiałkowska, E.A. Rosa, R.N. Bennett, Journal of Food Composition and Analysis, 23 (2010) 432.

[32] H. Horie, K. Kohata, Journal of Chromatography A, 881 (2000) 425.

[33] L.F. Wang, S.C. Park, J.O. Chung, J.H. Baik, S.K. Park, Journal of Food Science, 69 (2004) S301.

[34] S. Huang, C. Hung, W. Wu, B. Chen, Journal of Pharmaceutical and Biomedical Analysis, 48 (2008) 105.

[35] T.H. Kao, C.J. Chen, B.H. Chen, Talanta, 86 (2011) 349.

[36] R.n. Aparicio-Ruiz, M.I. Mínguez-Mosquera, B. Gandul-Rojas, Journal of Agricultural and Food Chemistry, 58 (2010) 6200.

[37] G.L. Roberston, D. Swinburne, Journal of Food Science, 46 (1981) 1557.

[38] S.J. Schwartz, T.V. Lorenzo, Critical Reviews in Food Science & Nutrition, 29 (1990) 1.

[39] M.P. Cano, Journal of Agricultural and Food Chemistry, 39 (1991) 1786.

[40] Council Directive 95/45, EC, (1995).

3.2. Characterisation of primary and secondary metabolites in processed fibres from juice industry

Recognizing the interest of the fruit industry in the characterisation of its commercial products, nine fibres obtained from juice industry by-products were selected. This chapter is focused on the characterisation and quantification of primary metabolites (sugars, organic acids, amino acids and fatty acids) and other secondary metabolites such as phenolic compounds, phytosterols, triterpenes, fatty alcohols, alkanes and carotenoids in these fibres. Until today, the characterisation of compounds in by-products has been performed over unprocessed materials or materials processed at laboratory scale [1, 2]. Here commercial by-products have been used. The nine selected fibres were apple, peach, pear, orange peel and flesh, tangerine peel and flesh, lemon flesh and carrot.

First, the phenolic compounds contained in these fibres were characterised and a comparison of its content and profile in lyophilised peel and in pulp from fresh fruit was established. There is extensive literature describing the effect of processing on polyphenols, but in most cases only addressed to the analysis of total polyphenols [3]. In this study we included an exhaustive analysis of different phenolic compounds and families present in the fruits from which the fibres were obtained to assess if any selective degradation of carotenoids occurred during processing as it has been described that each family of compounds may degrade differently during industrial processing. To analyse the individual phenolic compounds, reversed-phase high-performance liquid chromatography (HPLC) is commonly used to analyse the different groups of phenols. In this study an UPLC-PDA-FLR-MS/MS system was applied to quantify and identify the main extractable phenolic compounds in the studied fibre. At the same time, an optimised extraction with different solvent mixtures was applied to ensure the maximum recovery of phenolic family compounds [4]. The families found in the different fibres included hydroquinones, hydroxycinnamates, flavan-3-ols, flavonols, dihydrochalcones, flavones and flavanones.

Phenolic compounds such as proanthocyanidins, hydrolysable tannins, and phenolic acids, have been classified as bound or non-extractable. Few studies have addressed bound phenolic compounds in fruit and vegetables and their by-products [5]. We have compared the bound phenolic compounds present in the fibres taking into account those from the lyophilised fresh fruits. The objective was to know if fibre processing has any effect on them and if that effect is different to what is observed with the extractable phenolic compounds.

Another part of this study aimed to achieve a complete characterisation of the main metabolites of the fibres. Metabolite profile and content of the fibres obtained from juice industry by-products was compared with data reported in previous literature for the corresponding fresh fruit. In this study, primary metabolites such as sugars, sugar alcohols, amino acids, organic acids and fatty acids were included. Although the literature reports that during processing for juice production no degradation of these compounds occurs [6], they were included because steps such as wet milling and drying could affect the content of low chain organic acids, amino acids and sugars due to their high solubility in water. To analyse these compounds, a GC-MS methodology was applied after a previous derivatisation procedure [7].

On the other hand secondary metabolites such as phytosterols, triterpenes, fatty alcohols, alkanes and carotenoids were also analysed. Although these are present in much lower concentrations than the above mentioned metabolites, they have drawn a growing interest in recent years. Some studies demonstrate that these compounds have singular effects, suggesting that they may have an even greater role for human health than the already positive results seen to date [8, 9]. Then, the characterisation of these compounds in fibres becomes particularly important as these fibres could be converted into a product with added value. To analyse these compounds GC-MS methodology with previous derivatisation and UPLC-PDA-FLR-MS/MS was applied.

The results of these studies have been published on *Food Chemistry 172 (2015) 575-584* and submitted to *Food Composition and Analysis.*

References.

[1] A. Schieber, P. Keller, R. Carle, Journal of Chromatography A, 910 (2001) 265.

[2] H.H. Wijngaard, C. Rößle, N. Brunton, Food Chemistry, 116 (2009) 202.

[3] I. Volf, I. Ignat, M. Neamtu, V.I. Popa, Chemical Papers, 68 (2014) 121.

[4] I. Ignat, I. Volf, V.I. Popa, Food Chemistry, 126 (2011) 1821.

[5] S. Arranz, F. Saura-Calixto, S. Shaha, P.A. Kroon, Journal of Agricultural and Food Chemistry, 57 (2009) 7298.

[6] V. Gökmen, N. Artık, J. Acar, N. Kahraman, E. Poyrazoğlu, European Food Research and Technology, 213 (2001) 194.

[7] J. Lisec, N. Schauer, J. Kopka, L. Willmitzer, A.R. Fernie, Nature Protocols, 1 (2006) 387.

[8] G. Laufenberg, B. Kunz, M. Nystroem, Bioresource Technology, 87 (2003) 167.

[9] F. Poiroux-Gonord, L.P. Bidel, A.-L. Fanciullino, H.l.n. Gautier, F.l. Lauri-Lopez, L. Urban, Journal of Agricultural and Food Chemistry, 58 (2010) 12065.

3.2.1. Characterisation of phenolic compounds in processed fibres from the juice industry

CHARACTERISATION OF PHENOLIC COMPOUNDS IN PROCESSED FIBRES FROM THE JUICE INDUSTRY

Antoni Delpino-Rius[a], Jordi Eras[a,b,*], Francisca Vilaró[b], Miguel Ángel Cubero[c,d], Mercè Balcells[a,*,] Ramon Canela-Garayoa[a].

[a] Chemistry Department, ETSEA, University of Lleida, 25198-Lleida, Spain

[b] Scientific Technical Service DATCEM, University of Lleida, 25198-Lleida, Spain

[c] Food Science and Technology Department, University of Lleida, 25198-Lleida, Spain

[d] Indulleida, S.A., N. 230, km 12, 25125-Alguaire, Lleida, Spain.

Food Chemistry 172 (2015) 575-584

Keywords: Fibers; industrially processed fibers; phenolic compounds; bound phenolic compounds; UPLC..

ABSTRACT

The content of phenolic compounds was determined in nine industrially processed fibres derived from the juice industry. Apple, peach, and pear as non-citrus fruit fibres were examined, as well as orange peel and flesh, tangerine peel and flesh, and lemon flesh as citrus fruit fibres, and carrot as vegetable fibre. The extractable phenolic profile of all fibres was obtained by UPLC-PDA-FLR-MS/MS. Forty phenolic compounds were identified and their concentrations determined. In addition, bound phenolic acids and proanthocyanidins were measured in solid residues in order to determine the phenolic compounds remaining. Also, to allow the comparison of the profiles and contents in the fresh fruit and fibres, we analysed extractable and bound phenolic compounds in lyophilised peel and pulp from fresh fruit. The profile and phenolic content of the fibres was similar to that of the fresh fruit, except for flavan-3-ols, which registered lower values.

1. Introduction.

The recovery, recycling, and upgrading of waste material is particularly relevant in the food and food processing industry, in which wastes, effluents, residues, and by-products can be recovered and often upgraded to useful higher value products [1]. In this regard, a growing awareness of the relationship between diet and health has led to changes in dietary habits, these accompanied by an increasing demand for healthier foods. In particular, the health attributes of by-products obtained from fruit and vegetable processing are exploited in the food production industry. Ingredients of added value holding anti-oxidant compounds can be developed from by-products generated by the juice industry. One of the most relevant properties of fruit fibres is their bioactive compound content, such as anti-oxidants [2]. In this context, some studies have reported the physical, chemical, and functional properties of fruit and vegetable by-products, highlighting their anti-oxidant potential [3, 4].

Bioactive compounds, such as phenolic compounds, have beneficial effects in degenerative diseases and exhibit protective effects against cardiovascular diseases and cancer. Such compounds are present in the human diet, mainly through the ingestion of fruit and vegetables. Total phenolic content of several dried and fresh fruit and vegetable by-products has been described [5, 6]. Freeze-dried and dried apple by-products [7] has already been described, also the profile of these anti-oxidants has also been studied in freeze-dried and air-dried apple pomace [8], [9]. However, little attention has been devoted to the potential of pear and peach by-products as sources of phenolic compounds. In this regard, only the total phenolic content in these matrices has been described [7, 10]. In citrus fruits, total phenolic content in dried orange by-products has been described by Crizel [11] and in somewhat more detail by Fernández-López [12]. Flavanones, hesperidin, and eriocitrin in waste from the industrial processing of lemons have been determined by Coll [13]. Furthermore, the total phenolic content has been reported in carrot by-products [14].

The analysis of phenolic compounds usually involves a solvent extraction step using a combination of aqueous and organic solvents. Solvent extraction can be assisted by extraction techniques such as microwave or ultrasounds [8, 15]. After extraction, in most articles the total phenolic content in by-products is commonly determined using the Folin−Ciocalteu reagent. However, this reagent interacts with other reducing non-phenolic substances (e.g. vitamin C), thus leading to an overestimation of the total phenolic content present [16]. To analyse the individual phenolic compounds reversed-phase high-performance liquid chromatography (HPLC) is commonly used to analyse the different groups of phenols. In recent times, ultra performance liquid chromatography (UPLC) has been applied to improve the analysis of phenolic compounds in various matrices [17]. The solid residues from solvent extraction are generally considered exhausted material with regard to phenolic compounds. However, significant amounts of these anti-oxidants remain in the solid residue from fruit and vegetables [18, 19]. Phenolic compounds such as proanthocyanidins, hydrolysable tannins, and phenolic acids, have been classified as bound or non-extractable. Few studies have addressed bound phenolic compounds in fruit and vegetable by-products, with only one paper describing the non-extractable proanthocyanidins in freeze-dried apple waste [20].

To date, the characterisation of phenolic compounds in by-products has been performed on unprocessed materials or materials with processes optimised at laboratory scale. However, after the processing undergone during the industrial production of juice or related products, the fruit and vegetable by-products are subjected to several steps to obtain commercial fibres. In addition, in order to minimise damage to bioactive substances, all these processes have to be optimised, in particular the drying temperature [7].

Here we report on the content of extractable and bound phenolic compounds in nine industrially processed fibres. The fibres were obtained from various parts of several fruits and also from carrot. All fibres were obtained from processed industrial raw materials obtained from an actual line of juice processing. Additionally, we analysed the phenolic content of lyophilised peel and pulp from fresh fruit. The content in fresh fruit and industrial fibres was compared. Finally, we examined the heterogeneity of phenolic compound content in various production batches of five industrially produced fibres. These values might be conditioned by factors such as the cultivar or variety, climate, stage of maturity, storage, and processing [7].

2. Material and methods.

2.1 Reagents, solvents and phenolic standards.

Methanol and acetone (HPLC grade purity) were supplied by J.T. Baker (Deventer, The Netherlands). The chromatographic solvents were acetonitrile (LC-MS grade), also from J.T. Baker, and water was purified in a Milli-Q system from Millipore (Bedford, MA, USA). The chromatographic eluent additive was acetic acid (HAc) (LC-MS grade), provided by Fluka (Sigma-Aldrich, Madrid, Spain), formic acid (HFor) and hydrochloric acid 37% (analytical grade), supplied by Merck. Sodium chloride was obtained from Fluka, sodium hydroxide from Panreac (Barcelona, Spain) and ascorbic acid from Acros (Pittsburgh, PA,USA).

Standards of phenolic compounds were supplied as follows: (+)-catechin, (−)-epicatechin, procyanidin B1, procyanidin B2, arbutin, eriodictyol-7-*O*-rutinoside, naringenin-7-*O*-rutinoside, naringenin, hesperetin-7-*O*-rutinoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-rutinoside, kaempferol-3-*O*-rutinoside, isorhamnetin-3-*O*-rutinoside by Extrasynthèse (Genay, France); and arbutin, gallic acid, phloretin-2'-*O*-β-glucoside, 5'-

caffeoylquinic acid, caffeic acid, *p*-coumaric acid, ferulic acid and sinapic acid by Sigma–Aldrich Chemie (Steinheim, Germany). All stock standard solutions of phenolic compounds were prepared in MeOH and stored at 80 ºC. Working solutions were prepared from stock solutions by sampling an aliquot and diluting it with the injection solvent H_2O (0.1 % HAc).

2.2. Samples.

A local juice company (Indulleida S.A., Alguaire (Lleida), Spain) provided the fibre samples from the following: apple (6), pear (5), peach (5), carrot (1), orange flesh (6), orange peel (1), tangerine flesh (1), tangerine peel (1), and lemon flesh (5). A picture of fibres and scheme of the production process is shown in Supplementary data.

We purchased approximately 1 kg of randomly chosen "Golden" apples, "Blanquilla pear, "Amarillo de septiembre" peaches, carrots, "Navelina" oranges, tangerines, and lemons, from a local market. After distilled water washing, the peel of all the fruits was immediately separated from the pulp. The pulp from each kind of fruit was homogenised in a blender (Grindomix GM 200; Retsch, Haan, Germany) at 5000 rpm for 2 min and ascorbic acid (ca. 10 g/kg) was added to prevent oxidation. The peel was dipped in liquid nitrogen and ground into a fine powder using a pre-chilled mortar and pestle. Next, the peel and pulp were immediately frozen at –80 ºC and lyophilised at –50 ºC, 1.1 Pa for 24 h in a Cryodos-50 lyophiliser (Telstar, Terrassa, Spain). Finally, the lyophilised samples were powdered and stored at –20 ° C until analysis.

2.3 Extraction methods.

Extractable phenolic compounds.

Samples (0.25 g) were first extracted with 5 mL of methanol/water (50:50) (acidified at 0.1%, v/v with acetic acid) and then twice with 5 mL of acetone:water (70:30) in a ultrasonic bath for 30 min. Extracted solutions were pooled, diluted 1:4 with Milli-Q water (acidified at 0.1%, v/v with acetic acid), and filtered through 0.20 µm PTFE filters. Solutions were kept at 6ºC until UPLC analysis. All solvents contained ascorbic acid (0.2 % w/v).

Bound phenolic compounds:

Basic hydrolysis of bound phenolic acids: The lyophilised solid material obtained from the previous extractions was hydrolysed directly following a method previously described but with some modifications [21]. Part of the remaining material (50 mg) was mixed with 1.5 mL of 2 M NaOH containing 10 mM EDTA and 1% ascorbic acid and then shaken (Eppendorf© Thermomixer Comfort, Hamburg, Germany) for

30 min at 45°C. The reaction mixture was acidified (*c.a.* pH 3) by adding 0.285 mL of 7.2 M HCl and centrifuged at 1400x*g* for 5 min (Hettich Eppendorf Centrifuge MIKRO 22 R; Germany).

Acidic hydrolysis of bound proanthocyanidins: For the determination of bound flavan-3-ols, approximately 10 mg of the lyophilised solid residue was incubated with 5 mL of *n*-butanol/HCl (95:5) and 200 μL of Ferric reagent (2% ferric ammonium sulfate in 2 M HCl) for 60 min [22]. After cooling the solution in ice water and centrifugation, absorbance was measured at λ_{max} = 550 nm. Total flavan-3-ol concentration was determined using cyanidin chloride as reference compound.

2.4 Chromatographic analysis.

UPLC-PDA-FLR parameters.

Ultra performance liquid chromatographic analysis was performed on a Waters ACQUITY UPLC™ system (Waters, Milford, MA, USA) consisting of an ACQUITY UPLC™ binary solvent manager and ACQUITY UPLC™ sample manager, coupled to a photodiode array detector ACQUITY UPLC™ PDA and fluorescence detector ACQUITY UPLC™ FLR. Compounds were separated with an ACQUITY UPLC™ HSS T3 column (1.8 μm; 2.1 mm × 150 mm) (Waters, Manchester, UK) using a mobile phase consisting of solvent A, H_2O (0.1% v/v HAc), and solvent B, ACN 100% (0.1% v/v HAc). The flow rate was 0.550 mL/min. The linear gradient was as follows: 0–1.89 min, 1% B, (isocratic); 1.89–17.84 min, 30% B, (linear gradient); 17.84–21.39 min, 5% B, (linear gradient); 21.39–21.56 min, 1% B, (linear gradient); and 21.56–25 min, 1% B, (isocratic). Weak and strong needle solvents were H_2O (0.1% v/v HAc) and MeOH, respectively. The injection volume was 20 μL in mode full loop, and the column was kept at 45°C whilst the temperature in the sample manager was maintained at 10°C.

HPLC-UV parameters.

High performance liquid chromatographic analysis was performed on a Waters Alliance 2695 separations module system, coupled to a dual channel λ absorbance (320, 280 nm) detector. Compounds were separated with an Waters Atlantis dC18 column (5 μm; 2.1 mm × 150 mm) and using a gradient system with the mobile phase consisting of solvent A, H_2O (0.1% v/v HFor) and solvent B, MeOH 100% (0.1% v/v HAc). The flow rate was 0.500 mL/min. The linear gradient was as follows: 0–0.5 min, 10% B, (isocratic); 1.89–17.84 min, 30% B, (linear gradient); 0.5–15 min, 30% B, (linear gradient); 15–25 min, 60% B, (linear gradient); 25–35 min, 80% B, (linear gradient); 35–40 min, 10% B, (linear gradient); and 40–45 min, 10% B, (isocratic). The injection volume was 10 μL and the column was kept at 30°C, whilst the sample temperature was maintained at 10°C. The average maximum pressure in the chromatographic system was 16.55 10^6 Pa.

UPLC-MS parameters.

MS analysis was carried out on a Waters ACQUITY TQD tandem quadrupole mass spectrometer (Waters, UK). The instrument was operated using an electrospray source (ESI) in positive and negative ion mode. The ESI parameters were as follows: capillary voltages of 3.5 kV and -2.5 kV in positive and negative mode, respectively; the source at 150 °C; desolvation temperature 500 °C; cone gas (nitrogen) flow 50L/h; and desolvation gas flow 800L/h. Flow injections of each individual standard were used to optimise the cone voltage and Multiple Reaction Monitoring (MRM) parameters. Collision-induced dissociation was achieved using argon at a flow rate of 0.15 mL/min in the collision cell. MS conditions of standard compounds with same aglycone were applied to identify compounds with non-available standards. MassLynx 4.1 software (Waters, USA) was used for data acquisition.

2.5 Identification and quantification.

Identification of phenolic compounds in fibres was based on the available standards and other tools to identify the maximum number of peaks. The chromatographic behaviour and UV-vis absorption spectra, together with published data on the main phenolic compounds in the fruit and vegetables addressed were used for identification purposes. Of note, the phenolic compound profiles of apples [23], pears [24], peaches [25, 26], oranges [27, 28], lemons [27] and carrots[29] have been described. Furthermore, using the molecular ion and fragmentation pathways of MS, we also confirmed the structures of the phenolic compounds found.

To quantify, the calibration curves were constructed by plotting chromatographic peak areas measured at the specific wavelengths of the following chemical standards: 284 nm for HMF; 320 nm for hydroxycinnamic acids; 283 nm for flavanones; 285 nm for dihydrochalcone; and 340 nm and 350 nm for flavonols (apigenin, isorhamnetin, kaempferol and quercetin glycosides). Fluorescence values for flavan-3-ols were measured at 310 nm (λ_{ex}=280). For compounds for which no standards were available, quantification was performed with a linear curve (mmol/L) of a standard with the same aglycone (chromophoric part) through the corresponding correction to the molecular weight. The chromatographic areas of phenolic compounds present in samples, measured at its specific wavelength, were used to quantify by interpolation. Repeatability of the procedure for each sample type was determined by the analysis of one sample in triplicate. The results are detailed in the Supplementary data.

3. Results and discussion.

3.1 Extractable phenolic compounds.

The phenolic profile of various fruit fibres were analysed using liquid chromatography. In this regard, we identified 40 phenolic compounds and determined their concentrations. To obtain a suitable separation of groups of phenolics, the main was to stress the benefit of UPLC above HPLC analysis of phenolic compounds. The convenience of UPLC was clearly proven in terms of analysis speed, less than 25 minutes compared with more than 100 minutes using HPLC with no loss of resolution [30](Figure 1). For five types of fibre sample, there were several production batches, all of which had been subjected to the same processing. To evaluate the phenolic profile and content of fibres from the juice industry, we simultaneously used the same extraction method to evaluate the lyophilised peel and flesh of representative samples of the fruits addressed. The latter were chosen on the basis of same or similar varieties and origin to the fruit used by the juice industry.

Phenolic compounds were identified on the basis of criteria described in Section 2.5 of Material and methods. Caffeoylquinic and p-coumaroylquinic acids were identified in apple flesh, as this material is a recognised source of these compounds [17]. The isomers 3, 4 and 5 of coumaroylquinic and caffeoylquinic acids were distinguished on basis of the product ion spectra from $[M-H]^-$ and relative intensities of their ions. The characteristic ions were 191, 173 and 163 m/z in coumaroylquinic acid isomers and 191, 179 and 173 m/z in caffeoylquinic acid isomers [31]. The hydroxycinnamate family was found in several fruits, both in lyophilised flesh and peel, and in fibres. Flavan-3-ols were identified from the standards and confirmed in the samples on the basis of their characteristic fragmentation ions. Fluorescence response allows a more selective and sensitive quantification of compounds (λ_{ex}=280; λ_{em}=310 nm). Flavonoids are a widespread family of phenolic compounds, and we had access to at least one standard of each of the major families present in the fruits addressed. UV-Vis and mass spectra of these standards provided an effective tool to differentiate the aglycone family and allowed us to quantify the flavonoids present. To tentative identification of compounds for which standards were not available, we followed the practical guidelines for the characterisation of glycosyl flavonoids by triple quadrupole MS [30, 32, 33]. Also, studies reporting the flavonoid profiles of the fruits addressed here were useful.

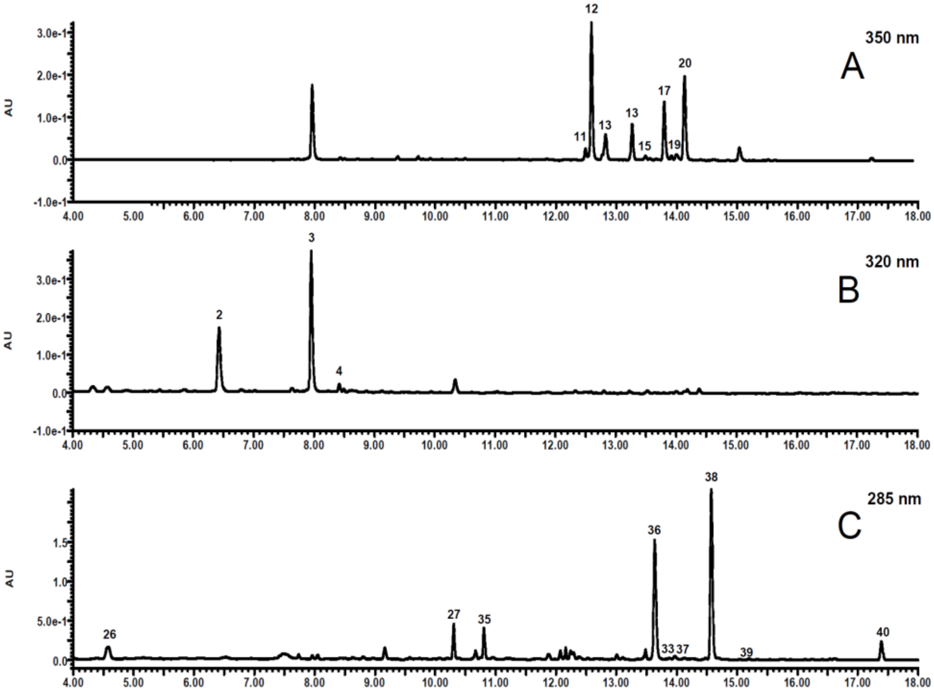


Figure 1. UPLC-PDA chromatograms of (A) apple fibre; (B) peach fibre; and (C) orange flesh fibre. Each chromatogram was generated by the combination of the characteristic wavelengths of the phenolic compounds analysed (280, 320 and 350 nm). Chromatographic peaks are numbered according to Tables 1a and 1b.

3.1.1 Phenolic compounds in fibre of non-citrus fruits and carrot.

Hydroxycinnamic acids, flavan-3-ols, and flavonols were found in the three non-citrus fruit fibres, namely apple, pear, and peach (Table1a). Peach showed the highest concentration of hydroxycinnamic acids (1587 μg/g vs. ~600 μg/g in apple and pear). 5-Caffeoylquinic acid (chlorogenic acid) was the main compound in this group. The content of other two isomers of caffeoylquinic (3 and 4-caffeoylquinic acids) was significant in peach fibres. Regarding the family of flavan-3-ols, the total content of these compounds in peach and apple fibres was around 200 μg/g, although the distribution differed in these two fruits. Epicatechin and procyanidin B2 were predominantly found in apple fibres, whereas catechin and procyanidin B1 were the main flavan-3-ols in those of peach. These profiles are consistent with that described in fresh fruit [23, 26]. Apple, especially apple peel, is an important source of flavonols [23]. In this regard, we observed 1839 μg/g flavonols in apple fibre, a value up to four times greater than that in pear fibres and up to 10 times greater than in peach fibres. The main flavonols in apple fibres were quercetin glycosides such as quercetin-3-O-galactoside (hyperoside) and quercetin-3-O-rhamnoside (quercitrin). We examined isorhamnetin derivatives in the three non-citrus fruit fibres and kaempferol-3-O-rutenoside in peach. In addition, around 300 μg/g of arbutin, a characteristic hydroquinone of pear [34], was found in pear fibres. Phloretin 2'-glucoside (phloridzin) was the predominant dihydrochalcone, together with 2'-xyloglucoside, in apple fibre, as has been described for fresh apples [23]. Phloridzin was also detected in carrot fibre, which registered the highest concentration (1798 μg/g). The fibre of this vegetable also showed the highest content of hydroxycinnamic acids (3001 μg/g distributed between caffeoylquinic acid isomers). Thus, carrot fibre is a good source of dihydrochalcones and hydroxycinnamic acids. Of note, our results are consistent with the phenolic profiles of apple pomace and by-products of the Pink Lady variety [9, 34, 35].

Lyophilised peel and flesh were extracted using the same procedure used for fibres, thus allowing direct comparison between the different materials. Figure 2 shows the results in terms of subclasses of phenolic compounds comparing fibres, lyophilised peel, and lyophilised flesh. The results for each phenolic compound in lyophilised samples are detailed in Supplementary data. In the three types of non-citrus fruit fibre, the hydroxycinnamic acid content did not differ greatly from that found in lyophilised peel and flesh. The flavonol concentration in lyophilised non-citrus fruit was higher in peel than in flesh, whilst fibres showed intermediate values. The same pattern was observed for hydroquinone in pear. In contrast, the content of flavan-3-ols in fibres was lower than in lyophilised peel and flesh in these three fruits. These results could be attributed to the degradation of flavan-3-ols during processing [9, 35]. Finally, the concentration of dihydrochalcones in apple and carrot were higher in fibre than in lyophilised peel and flesh; this finding is consistent with the phenolic profile of apple pomace of the Pink lady variety [9].

Table 1a. Contents of phenolic compounds in apple, pear, peach and carrot fibres determined using UPLC-PDA.

	R.t. (min)	Compound	Apple fibre	Pear fibre	Peach fibre	Carrot fibre
			Mean (µg/g, n=6) [a]	Mean (µg/g, n=5) [a]	Mean (µg/g, n=5) [a]	Mean (µg/g, n=1) [a]
	Hydroquinone					
1	2.53	Hydroquinone β-D-glucopyranoside		314±83		
				314±83		
	Hydroxycinnamates					
2	6.45	3-Caffeoylquinic acid		157±10	531±54	408
3	7.96	5-Caffeoylquinic acid	633.3±188.1	475±37	897.2±90.2	2024
4	8.43	4-Caffeoylquinic acid			158±5	508
5	9.47	5-Coumaroylquinic acid	8.2±1.5	1.5±2.1		32.8
6	9.72	4-Coumaroylquinic acid	26.8±10.3			26.2
			668±200	**634.4±49**	**1587±150**	**3000**
	Flavan-3-ols					
7	7.68	Procyanidin-B1			76.9±14.2	
8	8.22	(+)-Catechin			110±18	
9	9.23	Procyanidin-B2	126±62.9			
10	9.88	(-)-Epicatechin	89.7±43.9	29.5±4.5		
			216±107	**29.5±4.5**	**187±32**	
	Flavonols					
11	12.49	Quercetin-3-O-rutinoside	54.2±11.6	33.6±4	25.2±0.7	
12	12.59	Quercetin-3-O-galactoside	525±120	33.5±4.4	25.8±2.2	
13	12.82	Quercetin-3-O-glucoside	131±17	67±17	26.1±2.3	
15	13.27	Quercetin-derivate	140±20			
16	13.49	Quercetin-derivate	26.1±3			
17	13.64	Kaempferol 3-O-rutinoside			72.1±13.1	
18	13.8	Quercetin-derivate	203±37			
19	13.8	Isorhamnetin-3-O-robinioside		28.6±83.2		
20	13.98	Isorhamnetin-3-O-rutinoside	266±58	62.9±19.2	15.6±1.7	
21	14.12	Quercetin 3-O-rhamnoside	494±102			

Table 1a. Cont.

	R.t. (min)	**Compound**	**Apple fibre**	**Pear fibre**	**Peach fibre**	**Carrot fibre**
			Mean (µg/g, n=6) [a]	Mean (µg/g, n=5) [a]	Mean (µg/g, n=5) [a]	Mean (µg/g, n=1) [a]
22	14.12	Isorhamnetin-3-O-galactoside		30.4±8		
23	14.38	Isorhamnetin-3-O-glucoside		116±36		
24	15.36	Isorhamnetin 3-O-6"-malonylglucoside/galactoside		49.3±14.5		
			1839±370	**422±186**	**164±20**	
	Dihydrochalcones					
25	13.91	phloretin-2'-O-xyloglucoside	207±69			
26	15.03	phloretin 2'-O-glucoside	563±213			1768
			770±282			**1768**
	Total polyphenol content		**3493±958**	**1085±240**	**1939±202**	**4769**
27	4.3	5-(Hydroxymethyl)furfural (HMF)	122±43	85.4±28.8	106±57	42.4

[a] n corresponds to number of industrial batches

Table 1b. Contents of phenolic compounds in citrus fibres determined using UPLC-PDA.

	R.t. (min)	**Compound**	**Orange flesh fibre**	**Orange peel fibre**	**Tangerine flesh fibre**	**Tangerine peel fibre**	**Lemon flesh fibre**
			Mean (µg/g, n=6) [a]	**Mean (µg/g, n=1) [a]**	**Mean (µg/g, n=1) [a]**	**Mean (µg/g, n=1) [a]**	**Mean (µg/g, n=6) [a]**
	Hydroxycinnamates						
28	11.85	Ferulic acid	60.4±19.2	90.4	30.2	158	
			60.4±19.2	**90.4**	**30.2**	**158**	
	Flavones						
29	10.32	Apigenin 6,8-di-C-glucoside	506±321	738.6	208	961	44.4±5.8
30	11.05	Diosmetin-6,8-di-C-glucoside					121±10
31	12.69	Luteolin-7-O-rutenoside					45.1±4.6
32	13.29	Diosmetin-8-C-glucoside					77.3±9.5
33	13.49	Diosmetin-6-C-glucoside					64.1±2.2

Table 1b. Cont.

	R.t. (min)	**Compound**	**Orange flesh fibre**	**Orange peel fibre**	**Tangerine flesh fibre**	**Tangerine peel fibre**	**Lemon flesh fibre**
			Mean (µg/g, n=6) [a]	Mean (µg/g, n=1) [a]	Mean (µg/g, n=1) [a]	Mean (µg/g, n=1) [a]	Mean (µg/g, n=6) [a]
	Hydroxycinnamates						
28	11.85	Ferulic acid	60.4±19.2	90.4	30.2	158	
			60.4±19.2	**90.4**	**30.2**	**158**	
	Flavones						
29	10.32	Apigenin 6,8-di-C-glucoside	506±321	738.6	208	961	44.4±5.8
30	11.05	Diosmetin-6,8-di-C-glucoside					121±10
31	12.69	Luteolin-7-O-rutenoside					45.1±4.6
32	13.29	Diosmetin-8-C-glucoside					77.3±9.5
33	13.49	Diosmetin-6-C-glucoside					64.1±2.2
34	13.95	Apigenin-7-O-rutenoside	45.4±14.3		47.3	279.2	
			552±335	**738**	**256**	**1240**	**352±32**
	Flavanones						
35	10.64	Flavanone-derivate					135±9
36	12.26	Eriodictyol-7-O-rutinoside	148±41	280			1760±80
37	13.63	Naringenin-7-O-rutinoside	1936±1075	3111	871.8	3576	109±9
38	14.11	Naringenin-7-O-neohesperoside	37.8±14.9	26			58±30
39	14.59	Hesperetin-7-O-rutinoside	12377±4024	22339	5644	15821	4144±489
40	15.19	Hesperetin-7-O-glucoside	370±198	576		295	
41	17.41	Isosakuratenin-7-O-rutinoside	634±315	1253	330	1743	
			15503±5667	**27586**	**6847**	**21434**	**6205±617**
	Total polyphenol content		**16175±6041**	**28505**	**7163**	**22991**	**6557±649**
27	4.3	5-(Hydroxymethyl) furfural (HMF)	50.8±42.0	26.9	12.3	35.3	770±142

[a] n corresponds to number of industrial batches

3.1.2 Phenolic compounds in fibres of citrus fruits.

The phenolic profiles of citrus fruit fibres differed greatly from non-citrus materials (Table 1b). In this regard, the main phenolic compounds in citrus fruit fibres were flavones and flavanones. The profiles of tangerine and orange fibres were similar to those described for fresh fruit [27]. Hesperetin-7-rutenoside (hesperidin) and naringenin-7-O-rutenoside (narirutin) accounted for more than 90% of total phenolic content in these fibres. Other characteristic compounds of citrus fruits, such as isokuratenin-7-rutenoside (didymin), ferulic acid and apigenin derivatives, were also found. Although flesh and peel fibres from orange and tangerine showed the same phenolic profile, peel fibres showed much higher total phenolic compounds content than flesh. The profile and content of these compounds in orange fibres were similar to those described for non-industrial fibres processed on a pilot food plant [12]. Hesperidin and eriodictyol-7-rutenoside (eriocitrin) were the predominant phenolic compounds in lemon fibres, as they are in fresh lemon [27]. Apigenin, diosmetin and naringenin derivatives were found in minor amounts in this fibre. Indeed, our results are consistent with those found for the waste of industrially processed lemons[13].

The peel and flesh fibres of oranges and tangerines gave a similar phenolic profile to lyophilised peel and flesh. In both fibres and lyophilised material, flavones and flavanones showed a higher concentration in peel than in flesh (Figure 2). The flavone and flavanone content of the fibres and the lyophilised material did not differ greatly. This content was slightly higher for lyophilised orange peel and flesh, as well as for tangerine flesh. However, the content in tangerine peel fibre was slightly higher than in lyophilised peel. Lemon showed significantly lower concentrations of phenolic compounds in fibres than in peel and lyophilised flesh. These findings are consistent with the significantly greater content of 5-hydroxymethylfurfural (5-HMF) in lemon fibres (Table 1b), around 800 μg/g, which can be explained by degradation caused by the temperature reached during processing [36]. 5-HMF was not detected in the lyophilised samples.

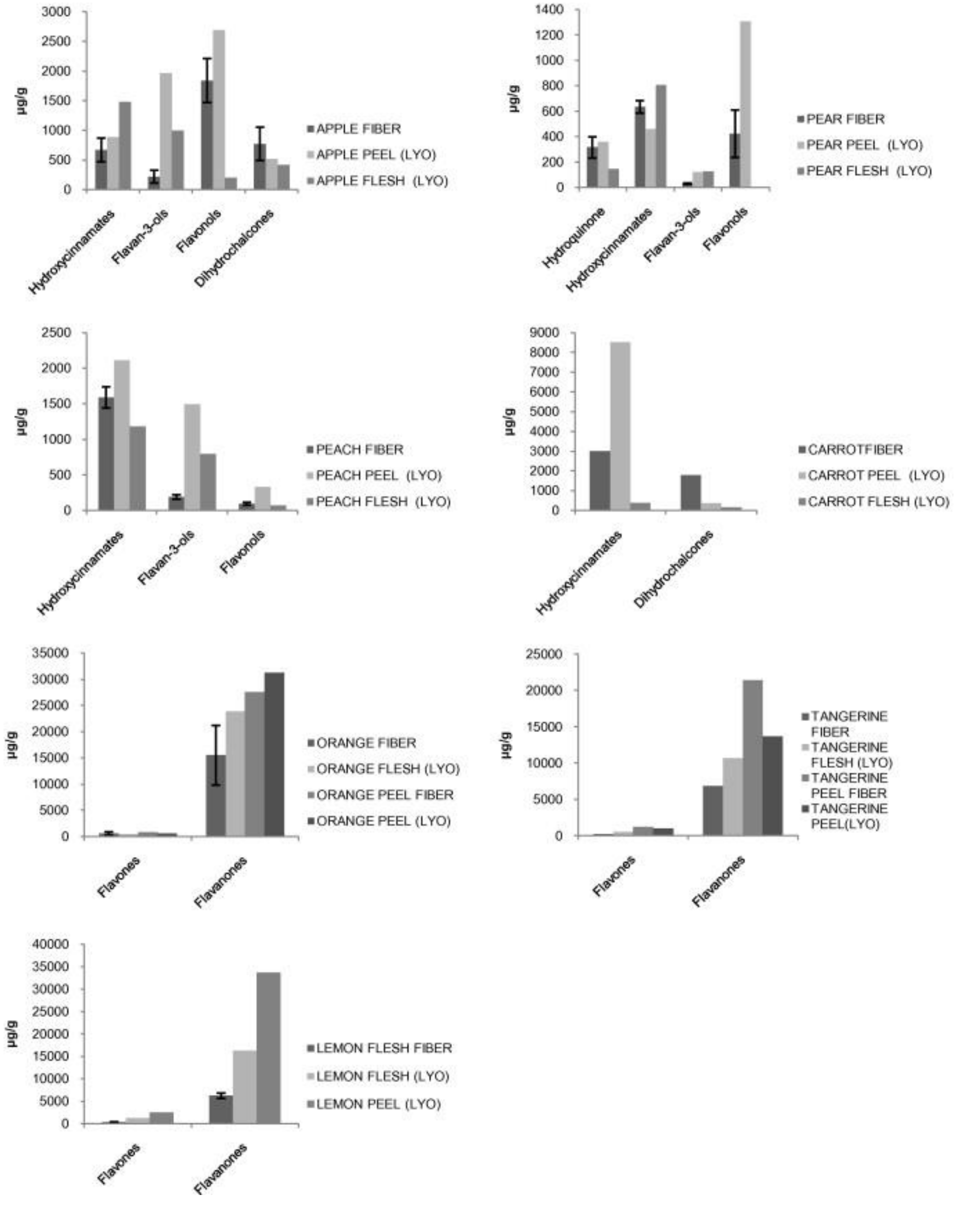


Figure 2. Phenolic compound content in fruit fibres and in lyophylysed peel and flesh. The results are grouped in the basis of phenolic families.

Table 2a. Phenolic acid content of fibres and lyophilised materials of *non Citrus* fruits determined using HPLC-PDA.

	Apple fibre	**Apple peel (LYO)**	**Apple flesh (LYO)**	**Pear fibre**	**Pear peel (LYO)**	**Pear flesh (LYO)**	**Peach fibre**	**Peach peel (LYO)**	**Peach flesh (LYO)**	**Carrot fibre**	**Carrot peel (LYO)**	**Carrot flesh (LYO)**
	Mean (µg/g, n=6)	Mean (µg/g, n=1)	Mean (µg/g, n=1)	Mean (µg/g, n=5)	Mean (µg/g, n=1)	Mean (µg/g, n=1)	Mean (µg/g, n=5)	Mean (µg/g, n=1)	Mean (µg/g, n=1)	Mean (µg/g, n=1)	Mean (µg/g, n=1)	Mean (µg/g, n=1)
Caffeic acid	12.9±4.7	16.3	23.9	6.2±1.3	13.3	5.4	6.2±3.7	13.3	5.4	47.5	51.7	20.2
Coumaric acid	8.9±2.1	11.3	4.1	1.7±0.36	1.8	1.1	2.9±0.7	1.8	1.1	2.3	2.6	0.9
Ferulic acid	3.1±0.5	2.3	2.0	1.3±0.1	1.0	1.9	1.4±0.2	1.0	1.9	7.2	7.9	4.1
Total Bound Phenolic Acids	24.9±7.4	29.9	30.0	9.2±1.8	16.2	8.5	10.5±4.47	16.2	8.5	57.0	62.1	25.2

n corresponds to number of samples

Table 2b. Phenolic acid content of fibres and lyophilised materials of *Citrus* fruits determined using HPLC-PDA.

	Orange flesh fibre	**Orange peel fibre**	**Orange peel (LYO)**	**Orange flesh (LYO)**	**Tangerine flesh fibre**	**Tangerine peel fibre**	**Tangerine peel (LYO)**	**Tangerine flesh (LYO)**	**Lemon flesh fibre**	**Lemon peel (LYO)**	Lemon flesh (LYO)
	Mean (µg/g, n=6)	Mean (µg/g, n=1)	Mean (µg/g, n=1)	Mean (µg/g, n=1)	Mean (µg/g, n=1)	Mean (µg/g, n=1)	Mean (µg/g, n=1)	Mean (µg/g, n=1)	Mean (µg/g, n=6)	Mean (µg/g, n=1)	Mean (µg/g, n=1)
Caffeic acid	63.4±18.0	35.7	7.3	37.0	58.1	29.9	45.6	17.6	9.4±1.8	73.8	39.9
Coumaric acid	9.2±1.3	31.5	39.8	8.1	8.9	31.6	172.1	5.7	6.5±0.7	21.8	10.5
Ferulic acid	36.9±4.5	89.8	71.1	38.4	30.1	91.0	498.9	54.6	6.1±0.6	10.7	5.7
Total Bound Phenolic Acids	109±23.8	157.0	118.3	83.5	97.0	152.4	716.7	78.0	21.9±0.3.1	106.3	56.1

n corresponds to number of samples

3.1.3 Total phenolic compound content.

In terms of total phenolic content calculated from the sum of the individual area of chromatographic peaks (Table 1a, 1b), the highest values, ranging from 7163 to 28505 µg/g, were found in orange and tangerine fibres from peel and flesh. However, the heterogeneity of batches (orange n=6) was also the highest, with 37 % of RSD, possibly caused by no specification of cultivars and provenance. The total phenolic compound content of lemon fibre was 6557 ± 649 µg/g. The total content of apple fibres (3493 ± 958 µg/g) was similar to that described in apple pomace and non-industrial waste fibres [9, 34, 35]. The total phenolic content of peach (1085 ± 240 µg/g) and pear (1939 ± 202 µg/g), lower than that of apple, is also consistent with reported values [37, 38]. Finally, the total phenolic content for carrot fibre was 4769 µg/g.

3.2 Bound phenolic acids.

The solid material remaining after the aqueous-organic extraction was subjected to alkaline hydrolysis and subsequently analysed by HPLC. To the best of our knowledge, the analysis of fruit fibres using this approach is unprecedented. Bound phenolic acids have been addressed in apple, nectarine, peach, tangerine peel, and young tangerine [18, 39], but these compounds were not studied separately in peel and pulp. Here we characterised caffeic, coumaric and ferulic acids (Table 2a) as they have been described as the main bound phenolic acids in fruit [15]. In apple, pear, peach, and carrot fibres, these acids registered concentrations ranging from 9.30 to 57.0 µg/g. The predominant bound phenolic acid was caffeic acid, the highest value found in carrot fibre (47.5 µg/g). Coumaric and ferulic acids were also assessed in these fibres. Apple fibre showed considerable concentrations of coumaric acid whilst carrot fibre showed a notable concentration of ferulic acid. The total content of bound phenolic acids in pear, peach, and carrot fibres fell between the corresponding contents in lyophilised peel and flesh. Total bound phenolic acid concentrations were slightly lower in apple fibre than in lyophilised peel and flesh. This profile was similar to that shown by extractable hydroxycinnamic acids, described in Section 3.1. For all the fruit and also for carrot, the amount of bound phenolic acids was higher in peel than in flesh. Of note, lyophilised peel and flesh showed a similar profile for bound phenolic acids.

Tangerine and orange fibres showed a concentration of bound phenolic acids ranging from 97.0 to 152.4 µg/g (Table 2b). These values were higher than those found in the other fibres. Caffeic acid was predominant in fibres from tangerine and orange flesh. In contrast, ferulic acid was most abundant in fibres from tangerine and orange peel. The total content of bound phenolic acids in fibres from tangerine and orange peel was higher than in flesh fibres. This behaviour was also observed between the lyophilised peel and flesh, which showed a higher concentration of ferulic acid in peel than in flesh. Finally, lemon fibre showed similar concentrations of coumaric, ferulic and caffeic acids. The concentrations of these compounds were significantly lower than those found in lyophilised materials; this behaviour was detected for the extractable phenolic compounds (Section 3.1). The industrial processing used also appears to favour the degradation of bound phenolic acids.

3.3 Bound proanthocyanidins (condensed tannins).

Whilst the determination of bound proanthocyanidins has been addressed by chromatography, thiolysis, or enzymatic treatments of residues, these approaches analysed only a small fraction of the total bound proanthocyanidins [19]. Spectrophotometric analysis of extracts obtained by treatment of the residues with n-butanol/HCl is the most common procedure for estimating the amount of bound proanthocyanidins in a matrix [22]. The proanthocyanidin content of the solid residues from the aqueous-organic extraction are expressed in cyanidin equivalents (Figure 3). All industrial fibres showed similar deviation percentage between batches (apple n=6, pear n=5 and peach n=5), ranging between 21.8 and 32.0%. Apple fibre showed the highest concentration of proanthocyanidins (30834 μg cyanidin/g), followed by peach and pear with 1536 and 1031 μg cyanidin/g, respectively. In the citrus and carrot fibres, the concentration of condensed tannins was considerably lower than in the other fibres. These results confirm those previously reported [15]. To the best of our knowledge, this study is the first to address bound proanthocyanidins separately in peel and flesh. Considering the lyophilised fruit, in apple the highest concentration of bound proanthocyanidins was found in peel, whilst in pear and peach it was detected in the flesh. Comparing fibre with lyophilised peel and flesh, the values for apple fibres were similar to those for lyophilised peel, whilst the values for pear fibre fell between those of the peel and flesh. Finally, the concentration of bound proanthocyanidins in peach fibre was lower than in lyophilised peel and flesh.

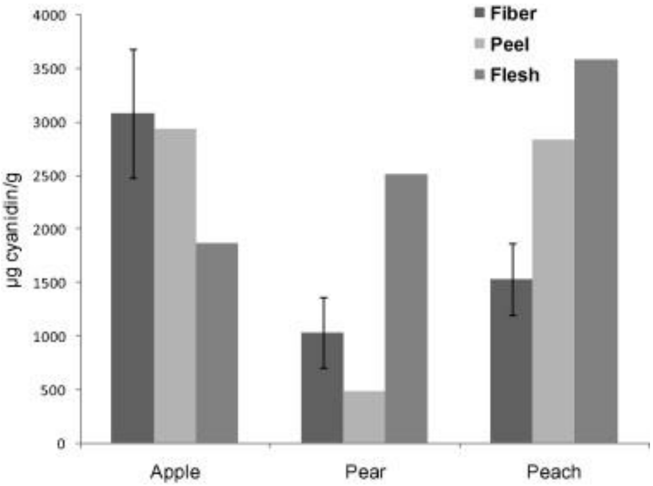


Figure 3. Bound proanthocyanidins content in the residues from aqueous-organic extraction expressed as cyanidin equivalents.

4. Conclusions.

Here we characterised the phenolic compounds of nine processed fibres from the juice industry, namely apple, peach, pear, orange peel and flesh, tangerine peel and flesh, lemon flesh and carrot. The extractable phenolic profiles of the fibres were achieved by UPLC-PDA-FLR-MS/MS. We identified 40 phenolic compounds in these fibres and determined their concentration. The phenolic profiles in fibres were found to be consistent with those in the lyophilised material from fresh fruits. In contrast, a different behaviour was observed when comparing industrial fibres with lyophilised peel and flesh, depending on the family of compounds assessed. In the case of non-citrus fruits, the content of flavan-3-ol was higher in fibres than in lyophilised peel and flesh of fresh fruit. Dihydrochalcones showed the opposite behaviour. In general, the content of flavanone and flavone content was slightly lower in citrus fibres than in peel and flesh. In addition, we examined bound phenolic compounds remaining in solid residues. Bound phenolic acids and bound proanthocyanidins differed in behaviour depending on the origin of the fibre or the lyophilised material. Given the health benefits of phenolic compounds, and on the basis of our findings, we conclude that the fibres from fruit and vegetable by-products show high added value as ingredients for the food manufacturing industry.

Acknowledgement.

The authors would like to thank Indulleida S.A. for providing samples. We also thank Tania Yates for the proofreading of the manuscript.

References.

[1] G. Laufenberg, B. Kunz, M. Nystroem, Bioresource Technology, 87 (2003) 167.

[2] J.A. Larrauri, Trends in Food Science & Technology, 10 (1999) 3.

[3] F. Figuerola, M.a.L. Hurtado, A.M.a. Estévez, I. Chiffelle, F. Asenjo, Food Chemistry, 91 (2005) 395.

[4] N. Grigelmo-Miguel, O. Martín-Belloso, LWT - Food Science and Technology, 32 (1999) 503.

[5] N. Balasundram, K. Sundram, S. Samman, Food Chemistry, 99 (2006) 191.

[6] N. O'Shea, E.K. Arendt, E. Gallagher, Innovative Food Science & Emerging Technologies, 16 (2012) 1.

[7] W. Peschel, F. Sánchez-Rabaneda, W. Diekmann, A. Plescher, I. Gartzía, D. Jiménez, R. Lamuela-Raventos, S. Buxaderas, C. Codina, Food Chemistry, 97 (2006) 137.

[8] A. Schieber, P. Keller, R. Carle, Journal of Chromatography A, 910 (2001) 265.

[9] H.H. Wijngaard, C. Rößle, N. Brunton, Food Chemistry, 116 (2009) 202.

[10] G.-F. Deng, C. Shen, X.-R. Xu, R.-D. Kuang, Y.-J. Guo, L.-S. Zeng, L.-L. Gao, X. Lin, J.-F. Xie, E.-Q. Xia, International Journal of Molecular Sciences, 13 (2012) 8308.

[11] T. de Moraes Crizel, A. Jablonski, A. de Oliveira Rios, R. Rech, S.H. Flôres, LWT - Food Science and Technology, 53 (2013) 9.

[12] J. Fernández-López, E. Sendra-Nadal, C. Navarro, E. Sayas, M. Viuda-Martos, J.A.P. Alvarez, International Journal of Food Science & Technology, 44 (2009) 748.

[13] M.D. Coll, L. Coll, J. Laencina, F.A. Tomás-Barberán, Zeitschrift für Lebensmitteluntersuchung und -Forschung A, 206 (1998) 404.

[14] P. Chantaro, S. Devahastin, N. Chiewchan, LWT - Food Science and Technology, 41 (2008) 1987.

[15] I. Ignat, I. Volf, V.I. Popa, Food Chemistry, 126 (2011) 1821.

[16] S. George, P. Brat, P. Alter, M.J. Amiot, Journal of Agricultural and Food Chemistry, 53 (2005) 1370.

[17] K.M. Kalili, A. de Villiers, Journal of Separation Science, 34 (2011) 854.

[18] S. Arranz, F. Saura-Calixto, S. Shaha, P.A. Kroon, Journal of Agricultural and Food Chemistry, 57 (2009) 7298.

[19] J. Pérez-Jiménez, J.L. Torres, Journal of Agricultural and Food Chemistry, 59 (2011) 12713.

[20] W.W. Tow, R. Premier, H. Jing, S. Ajlouni, Journal of food science, 76 (2011) T163.

[21] M. Nardini, E. Cirillo, F. Natella, D. Mencarelli, A. Comisso, C. Scaccini, Food Chemistry, 79 (2002) 119.

[22] L. Porter, Australian Journal of Chemistry, 39 (1986) 557.

[23] R. Tsao, R. Yang, J.C. Young, H. Zhu, Journal of Agricultural and Food Chemistry, 51 (2003) 6347.

[24] L.-Z. Lin, J.M. Harnly, Journal of Agricultural and Food Chemistry, 56 (2008) 9094.

[25] M.I. Gil, F.A. Tomás-Barberán, B. Hess-Pierce, A.A. Kader, Journal of Agricultural and Food Chemistry, 50 (2002) 4976.

[26] M. Scordino, L. Sabatino, A. Muratore, A. Belligno, G. Gagliano, Journal of Food Quality, 35 (2012) 255.

[27] B. Abad-García, S. Garmón-Lobato, L.A. Berrueta, B. Gallo, F. Vicente, Talanta, 99 (2012) 213.

[28] M.C. Díaz-García, J.M. Obón, M.R. Castellar, J. Collado, M. Alacid, Food Chemistry, 138 (2013) 938.

[29] D. Zhang, Y. Hamauzu, Journal: Food, Agriculture and Environment, 2 (2004) 95.

[30] B. Abad-Garcia, L.A. Berrueta, S. Garmon-Lobato, B. Gallo, F. Vicente, J Chromatogr A, 1216 (2009) 5398.

[31] M.N. Clifford, K.L. Johnston, S. Knight, N. Kuhnert, Journal of Agricultural and Food Chemistry, 51 (2003) 2900.

[32] B. Abad-Garcia, S. Garmon-Lobato, L.A. Berrueta, B. Gallo, F. Vicente, J Mass Spectrometry, 44 (2009) 1017.

[33] U. Vrhovsek, D. Masuero, M. Gasperotti, P. Franceschi, L. Caputi, R. Viola, F. Mattivi, Journal of Agricultural and Food Chemistry, 60 (2012) 8831.

[34] A. Schieber, F.C. Stintzing, R. Carle, Trends in Food Science and Technology, 12 (2001) 401.

[35] A. Schieber, P. Hilt, P. Streker, H.-U. Endreß, C. Rentschler, R. Carle, Innovative Food Science & Emerging Technologies, 4 (2003) 99.

[36] A. Del Caro, A. Piga, I. Pinna, P.M. Fenu, M. Agabbio, Journal of Agricultural and Food Chemistry, 52 (2004) 4780.

[37] I.H. Adil, H. Cetin, M. Yener, A. Bayındırlı, The Journal of Supercritical Fluids, 43 (2007) 55.

[38] D. Ferreira, S. Guyot, N. Marnet, I. Delgadillo, C.M. Renard, M.A. Coimbra, Journal of Agricultural and Food Chemistry, 50 (2002) 4537.

[39] X.-Q. Ye, J.-C. Chen, D.-H. Liu, P. Jiang, J. Shi, S. Xue, D. Wu, J.-G. Xu, Y. Kakuda, Food Chemistry, 124 (2011) 1561.

APPENDIX A. Supplementary data.

Picture of various fibres assessed.

Figure S-1. Picture of fibres analysed in the study.

Scheme of the production process.

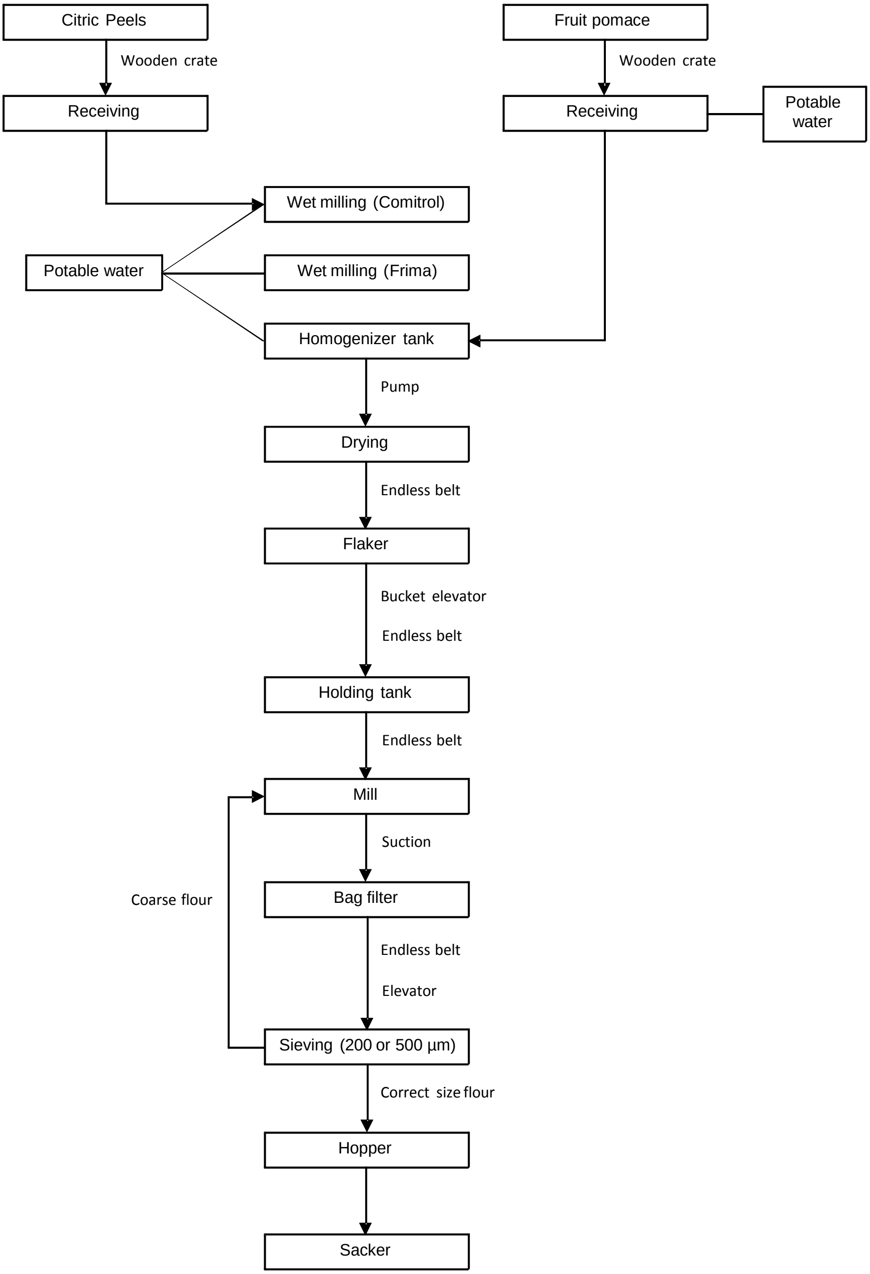


Figure S-2. Process flow to obtain fruit or vegetable fibres from juice industry by-products.

Detailed chromatographic, UV-vis, and MS parameters and bibliographic references of the phenolic compounds found in the samples.

References cited in table S-1.

1. Clifford, M.N., et al., Journal of Agricultural and Food Chemistry, 2003. 51(10): p. 2900-2911.
2. Kalili, K.M. and A. de Villiers, Journal of Separation Science, 2011. 34(8): p. 854-876.
3. Lin, L.-Z. and J.M. Harnly, Journal of Agricultural and Food Chemistry, 2008. 56(19): p. 9094-9101.
4. Abad-Garcia, B., et al., Journal of Chromatography A, 2009. 1216(28): p. 5398-415.
5. Abad-Garcia, B., et al., Journal of Mass Spectrometry, 2009. 44(7): p. 1017-25.
6. Abad-García, B., et al., Talanta, 2012. 99(0): p. 213-224.

Table S-1. Chromatographic, UV-vis, and MS parameters of the phenolic compounds found in the samples.

Peak No.	R.t. (min)	Identification	UV max (nm)	[M-H]$^-$ /[M+H]$^+$ (m/z)	Relative abundances of main ions detected from MS/MS of [M-H]-/[M+H]+	CE (eV)	Ref.
	Hydroquinone						
1	2.53	Hydroquinone β-D-glucopyranoside ^{a}	283	271/273	-/108(100), 95 (18)	20	
	Hydroxycinnamates						
2	6.45	3-caffeoylquinic acid ^{b}	240, 326	353/355	-/191 (100), 179 (2), 171(1)	20	[1]
3	7.96	5-caffeoylquinic acid ^{a}	240, 326	353/356	163(100), 145(62), 135(20), 117(20)/191 (100), 179 (94)	20	
4	8.43	4-caffeoylquinic acid ^{b}	240, 326	353/357	-/173 (100), 179(67),191(32)	20	[1]
5	9.47	5-coumaroylquinic acid ^{b}	235,312	337/339	-/191(100), 163(22), 191(173)	20	[1]
6	9.72	4-coumaroylquinic acid ^{b}	235,312	337/340	-/173 (100), 163(7),191(1)	20	[1]
27	11.85	Ferulic acid ^{a}	321	193/195	-/-	20	
	Flavan-3-ols						
7	7.68	Procyanidin-B1 ^{a}	278	289/291	291(100), 409(88), 427(64), 247(64), 301(64) /407(100), 289(63), 425 (34)	20	
8	8.22	(+)-Catechin ^{a}	278	289/291	139(100), 123(47), 147(22)/203(100), 109(94), 125(84)	20	
9	9.23	Procyanidin-B2 ^{a}	278	577/579	291(100), 409(88), 427(64), 247(64), 301(64) /407(100), 289(63), 425 (34)	20	
10	9.88	(-)-Epicatechin ^{a}	278	577/580	139(100), 123(47), 147(22)/203(100), 109(94), 125(84)	20	
	Flavonols						
11	12.49	quercetin-3-O-rutinoside ^{a}	255, 353	609/611	303(100)/300(100)	30	

Table S-1. Cont.

Peak No.	**R.t. (min)**	**Identification**	**UV max (nm)**	**$[M-H]^-$/$[M+H]^+$ (m/z)**	**Relative abundances of main ions detected from MS/MS of [M-H]-/[M+H]+**	**CE (eV)**	**Ref.**
12	12.59	quercetin-3-O-galactoside [a]	255, 353	463/465	303(100), 153(26)/300(100), 271(27), 255(19)	30	
13	12.82	quercetin-3-O-glucoside [a]	255, 353	463/465	303(100), 153(26)/300(100), 271(27), 255(19)	30	
14	13.27	quercetin-derivate [b]	255, 353	433/435	303(100)/-	30	[2]
15	13.49	quercetin-derivate [b]	255, 353	433/435	303(100)/-	30	[2]
16	13.64	Kaempferol 3-O-rutinoside [a]	265, 347	593/595	287/-	30	
17	13.8	quercetin-derivate [b]	255, 353	433/435	303(100)/-	30	[1]
18	13.8	isorhamnetin-3-O-robinioside [b]	254, 352	623/625	317/-	30	[3,4]
19	13.98	isorhamnetin-3-O-rutinoside [a]	254, 352	623/625	317/-	30	
20	14.12	quercetin 3-O-rhamnoside [b]	255, 353	447/449	303(100), 153(26)/300(100), 271(27), 255(19)	30	
21	14.12	isorhamnetin-3-O-galactoside [b]	254, 352	477/479	317/-	30	[3,4]
22	14.38	isorhamnetin-3-O-glucoside [b]	254, 352	477/479	317/-	30	[3,4]
23	15.36	Isorhamnetin 3-O-6''-malonygalactoside [b]	254, 352	563/565	317/-	30	[3,4]
	Dihydrochalcones						
24	13.91	phloretin-2'-O-xyloglucoside [b]	285	567/569	-/167(100), 275(29), 125(29)	30	[2,3]

TableS-1. Cont.

Peak No.	R.t. (min)		Identification	UV max (nm)	[M-H]$^-$/[M+H]$^+$ (m/z)	Relative abundances of main ions detected from MS/MS of [M-H]-/[M+H]+	CE (eV)	Ref.
25	15.03		phloretin 2'-O-glucoside [a]	285	433/435	-/167(100), 275(29), 125(29)	30	
Flavones								
28	10.32		Apigenin 6,8-di-C-glucoside [b]	267, 340	593/595	-/325(100), 409(82), 337(67), 295(65), 427(56), 379(56), 355(56), 439(56), 403(36), 523(34), 390(34), 457(36)	30	[5, 6]
29	11.05		Diosmetin-6,8-di-C-glucoside [b]	271, 347	623/625	-/487(100), 409(93), 355(87), 385(36), 456(36), 367(36)	30	[5, 6]
30	12.69		Luteolin-7-O-rutenoside [b]	268, 348	593/595	-/287(100)	30	[5, 6]
31	13.29		Diosmetin-8-C-glucoside [b]	271, 347	461/463	-/343(100), 313(58), 367(42), 353(25)	30	[5, 6]
32	13.49		Diosmetin-6-C-glucoside [b]	271, 347	461/463	-/313(100), 343(90), 367(34), 353(16)	30	[5, 6]
33	13.95		Apigenin-7-O-rutenoside [b]	267, 340	577/579	-/258(100)	30	[5, 6]
Flavanones								
34	10.64		Flavanone-derivate	284	651/653	-/153(100)	30	[5, 6]
35	12.26		Eriodictyol-7-O-rutinoside [a]	284	595/597	-/435(100), 289(90), 450(34)	30	
36	13.63		Naringenin-7-O-rutinoside [a]	284	579/581	-/273(100)	30	
37	14.11		Naringenin-7-O-neohesperoside [b]	284	579/581	-/273(100)	30	[5, 6]
38	14.59		Hesperetin-7-O-rutinoside [a]	284	609/611	-/303(100), 449(87)	30	

Table S-1. Cont.

Peak No.	R.t. (min)	Identification	UV max (nm)	$[M-H]^-$ / $[M+H]^+$ (m/z)	Relative abundances of main ions detected from MS/MS of [M-H]-/[M+H]+	CE (eV)	Ref.
39	15.19	Hesperetin-7-O-glucoside [b]	284	463/465	-/303(100), 449(37)	30	[5, 6]
40	17.41	Isosakuratenin-7-O-rutinoside [b]	284	593/595	-/287(100), 433(1009	30	[5, 6]

[a] Identification considering the UV-vis spectrum and specific mass fragments of the standard compounds.

[b] Tentative identification based on UV-vis spectrum, MS, and HPLC/UHPLC retention times and published data.

Detailed result tables of extractable and bound phenolic compounds in fibres and lyophilised materials.

Table S-2. Results of phenolic compounds of non-citrus fruits (Apple).

		APPLE FIBRE			**APPLE PEEL**		**APPLE FLESH**	
R.t	**Compound**	**Conc.**	**Batch SD**	**RSD[a]**	**Conc.**	**RSD[a]**	**Conc.**	**RSD[a]**
Hydroxycinnamates								
7.96	5-Caffeoylquinic acid	633.3	188.1	2.2	829.8	2.9	1468.7	2.7
9.47	5-Coumaroylquinic acid	8.2	1.5	0.8	10.8	5.0	13.2	3.3
9.72	4-Coumaroylquinic acid	26.8	10.3	0.7	39.7	1.2		
		668.3	199.9		880.3		1481.9	
Flavan-3-ols								
7.68	Procyanidin-B1				90.1	1.8	183.7	0.4
8.22	(+)-Catechin				36.3	1.3	47.7	1.3
9.23	Procyanidin-B2	126.0	62.9	2.6	1117.3	0.2	473.9	0.5
9.88	(-)-Epicatechin	89.7	43.9	0.4	719.7	0.2	292.0	0.3
Total		215.7	106.8		1963.4		997.3	
Flavonols								
12.49	Quercetin-3-O-rutinoside	54.2	11.6	1.1	45.2	1.5	32.8	0.0
12.59	Quercetin-3-O-galactoside	525.5	120.2	0.3	463.1	2.1	45.2	1.6
12.82	Quercetin-3-O-glucoside	130.7	16.6	0.8	121.9	2.1	26.0	1.8
13.27	Quercetin-derivate	140.0	20.4	1.3	176.2	1.1	27.7	1.3
13.49	Quercetin-derivate	26.1	3.0	4.3	33.4	1.8		
13.8	Quercetin-derivate	202.7	37.1	0.5	287.3	0.9		
13.98	Isorhamnetin-3-O-rutinoside	266.2	58.4	74.4	334.3	2.0		
14.12	Quercetin 3-O-rhamnoside	493.6	102.2	0.4	1231.3	2.1	68.8	0.6
Total		1839.0	369.5		2692.7		200.5	

[a] Repeatability of the procedure for each sample type was determined by the analysis of one sample in triplicate.

Table S-2. Cont.

		APPLE FIBRE			APPLE PEEL		APPLE FLESH	
R.t	**Compound**	**Conc.**	**Batch SD**	**RSD[a]**	**Conc.**	**RSD[a]**	**Conc .**	**RSD[a]**
Dihydrochalcones								
13.91	phloretin-2'-O-xyloglucoside	207.4	69.4	1.0	307.3	0.6	200.4	0.8
15.03	phloretin 2'-O-glucoside	562.9	212.8	2.1	203.7	0.6	215.8	0.7
Total		770.3	282.2		511.0		416.2	

[a] Repeatability of the procedure for each sample type was determined by the analysis of one sample in triplicate.

Table S-3. Results of phenolic compounds of non-citrus fruits (Pear).

		PEAR FIBRE			PEAR PEEL		PEAR FLESH	
R.t	**Compound**	**Conc.**	**Batch SD**	**RSD[a]**	**Conc.**	**RSD[a]**	**Conc.**	**RSD[a]**
Hydroquinone								
2.53	Hydroquinone β-D-glucopyranoside	314.4	83.2	2.6	356.5	1.5	142.3	0.3
Total		314.4	83.2		356.5		142.3	
Hydroxycinnamates								
6.45	3-Caffeoylquinic acid	157.9	10.3	0.2	152.7	0.7	141.6	1.0
7.96	5-Caffeoylquinic acid	475	37.1	0.9	305.6	5.1	665	2.5
9.47	5-Coumaroylquinic acid	1.5	2.1	7.2				
Total		634.4	49.5		458.3		806.6	
Flavan-3-ols								
9.23	Procyanidin-B2				39.3	1.6	61	1.0
9.88	(-)-Epicatechin	29.5	4.5	1.5	80.4	0.3	63.3	0.3
Total		29.5	4.5		119.7		124.3	
Flavonols								
12.49	Quercetin-3-O-rutinoside	33.6	4	3.2	175	0.7		
12.59	Quercetin-3-O-galactoside	33.5	4.4	1.3	330.6	1.2		
12.82	Quercetin-3-O-glucoside	66.9	16.5	3.4	357.5	1.7		
13.8	Isorhamnetin-3-O-robinioside	28.6	83.2	1.3	124.6	0.8		

[a] Repeatability of the procedure for each sample type was determined by the analysis of one sample in triplicate.

Table S-3. Cont.

		PEAR FIBRE			PEAR PEEL		PEAR FLESH	
R.t	**Compound**	**Conc.**	**Batch SD**	**RSD[a]**	**Conc.**	**RSD[a]**	**Conc.**	**RSD[a]**
13.98	Isorhamnetin-3-O-rutinoside	62.9	19.2	2.3	52.9	2.1		
14.12	Isorhamnetin-3-O-galactoside	30.4	8	2.5	40.3	6.6		
14.38	Isorhamnetin-3-O-glucoside	116.4	36.4	2.0	73	0.8		
15.36	Isorhamnetin 3-O-6"-malonylglucoside/ galactoside	49.3	14.5	3.1	154.6	2.2		
Total		421.6	186.2		1308.5			

[a] Repeatability of the procedure for each sample type was determined by the analysis of one sample in triplicate.

Table S-4. Results of phenolic compounds of non-citrus fruits (Peach).

		PEACH FIBRE			PEACH PEEL		PEACH FLESH	
R.t	**Compound**	**Conc.**	**Batch SD**	**RSD[a]**	**Conc.**	**RSD[a]**	**Conc.**	**RSD[a]**
Hydroxycinnamates								
6.45	3-Caffeoylquinic acid	531.2	54.4	3.8	436	2.5	486.3	1.7
7.96	5-Caffeoylquinic acid	897.2	90.2	3.5	1515.8	2.4	551.7	1.5
8.43	4-Caffeoylquinic acid	158.7	5	1.8	156	0.8	140.2	0.3
Total		1587.1	149.6		2107.8		1178.2	
Flavan-3-ols								
7.68	Procyanidin-B1	76.9	14.2	1.4	594.8	1.0	338	1.2
8.22	(+)-Catechin	110.1	18.1	3.6	896.2	1.4	454	1.3
Total		187.0	32.3		1491.0		792.0	
Flavonols								
12.49	Quercetin-3-O-rutinoside	25.2	0.7	1.8	76	5.8	18.2	2.8
12.59	Quercetin-3-O-galactoside	25.8	2.2	1.1	97.7	8.7	19.3	2.1

[a] Repeatability of the procedure for each sample type was determined by the analysis of one sample in triplicate.

[a] Repeatability of the procedure for each sample type was determined by the analysis of one sample in triplicate

Table S-4. Cont.

		PEACH FIBRE			**PEACH PEEL**		**PEACH FLESH**	
R.t	**Compound**	**Conc.**	**Batch SD**	**RSD[a]**	**Conc.**	**RSD[a]**	**Conc.**	**RSD[a]**
12.82	Quercetin-3-O-glucoside	26.1	2.3	2.6	94	9.0	17.7	4.0
13.64	Kaempferol-3-O-rutinoside	72.1	13.1	9.8	197	1.3		
13.98	Isorhamnetin-3-O-rutinoside	15.6	1.7	4.1	58.5	5.1	7.8	7.8
Total		164.8	20.0		523.2		63.0	

[a] Repeatability of the procedure for each sample type was determined by the analysis of one sample in triplicate.

Table S-5. Results of phenolic compounds of non-citrus fruits (Carrot).

		CARROT FIBRE		**CARROT PEEL**		**CARROT FLESH**	
R.t	**Compound**	**Conc.**	**RSD[a]**	**Conc.**	**RSD[a]**	**Conc.**	**RSD[a]**
Hydroxycinnamates							
6.45	3-Caffeoylquinic acid	408.2	1.5				
7.96	5-Caffeoylquinic acid	2024.9	1.0	8369.6	1.2	353.9	0.9
8.43	4-Caffeoylquinic acid	508.6	0.2				
9.47	5-Coumaroylquinic acid	32.8	0.5	142.3	0.2	17.6	1.0
9.72	4-Coumaroylquinic acid	26.2	5.0				
Total		3000.7		8511.9		371.5	
Dihydrochalcones							
15.03	phloretin 2'-O-glucoside	1768.3	1.7				
Total		1768.3					

[a] Repeatability of the procedure for each sample type was determined by the analysis of one sample in triplicate.

Table S-6. Results of phenolic compounds of citrus fruits (Orange).

		ORANGE FIBER			**ORANGE PEEL**		**ORANGE FLESH**	
R.t	**Compound**	**Conc.**	**Batch SD**	**RSD[a]**	**Conc.**	**RSD[a]**	**Conc.**	**RSD[a]**
Hydroxycinnamates								
11.85	Ferulic acid	60.4	19.2	2.1	8.4	0.0	15.8	5.0
		60.4	19.2		8.4		15.8	

[a] Repeatability of the procedure for each sample type was determined by the analysis of one sample in triplicate.

Table S-6. Cont.

		ORANGE FIBER			ORANGE PEEL		ORANGE FLESH	
R.t	**Compound**	**Conc.**	**Batch SD**	**RSD[a]**	**Conc.**	**RSD[a]**	**Conc.**	**RSD[a]**
Flavones								
10.32	Apigenin 6,8-di-C-glucoside	506.2	321.1	2.2	562.5	3.8	413.4	4.7
13.95	Apigenin-7-O-rutenoside	45.4	14.3	5.6				
Total		551.6	335.4		562.5		413.4	
Flavanones								
12.26	Eriodictyol-7-O-rutinoside	148.1	40.6	3.0	536	4.1	217.5	6.9
13.63	Naringenin-7-O-rutinoside	1935.7	1074.7	1.8	1488.4	3.1	1583.7	4.2
14.1	Naringenin-7-O-neohesperoside	37.8	14.9	6.5	26.7	8.2	9.3	17.9
14.59	Hesperetin-7-O-rutinoside	12377.1	4023.9	1.6	27551	1.5	20541.1	2.0
15.19	Hesperetin-7-O-glucoside	370.3	197.5	0.6	612.2	3.9	275.1	4.6
17.41	Isosakuratenin-7-O-rutinoside	634	315.4	2.6	1012.3	1.0	1158.4	0.6
Total		15503	5667		31226.6		23785.1	

[a] Repeatability of the procedure for each sample type was determined by the analysis of one sample in triplicate.

Table S-7. Results of phenolic compounds of citrus fruits (Tangerine).

		TANGERINE FIBER		TANGERINE PEEL		TANGERINE FLESH		TANGERINE PEEL FIBER	
R.t	**Compound**	**Conc.**	**RSD[a]**	**Conc.**	**RSD[a]**	**Conc.**	**RSD[a]**	**Conc.**	**RSD[a]**
Hydroxycinnamates									
11.85	Ferulic acid	30.2	1.7	45.9	6.6	15.3	3.2	79.2	8.1
Total		30.2		45.9		15.3		79.2	
Flavones									
10.32	Apigenin 6,8-di-C-glucoside	208.7	2.2	815	0.9	464.8	1.2	480.3	2.5
13.95	Apigenin-7-O-rutenoside	47.3	2.3	166.7	2.8	101.2	3.1	139.6	7.6
Total		256		981.7		566		619.9	
Flavanones									

[a] Repeatability of the procedure for each sample type was determined by the analysis of one sample in triplicate.

		TANGERINE FIBER		**TANGERINE PEEL**		**TANGERINE FLESH**		**TANGERINE PEEL FIBER**	
R.t	**Compound**	**Conc.**	**RSD[a]**	**Conc.**	**RSD[a]**	**Conc.**	**RSD[a]**	**Conc.**	**RSD[a]**
13.63	Naringenin-7-O-rutinoside	871.8	0.6	925	5.8	915.2		1788.2	7.6
14.59	Hesperetin-7-O-rutinoside	5644.4	0.5	12318	0.6	9422		7910.3	7.6
15.19	Hesperetin-7-O-glucoside			161.2	0.7			147.3	6.1
17.41	Isosakuratenin-7-O-rutinoside	330.4	3.0	283	2.5	340.1		871.3	7.0
Total		6846.6		13687.2		10678		10717.1	

Table S-8. Results of phenolic compounds of citrus fruits (Lemon).

		LEMON FIBER			**LEMON PEEL**		**LEMON FLESH**	
R.t	**Compound**	**Conc.**	**Batch SD**	**RSD[a]**	**Conc.**	**RSD[a]**	**Conc.**	**RSD[a]**
Flavones								
10.32	Apigenin 6,8-di-C-glucoside	44.4	5.8	4.8	1005.5	3.2	235.4	6.6
11.05	Diosmetin-6,8-di-C-glucoside	121.3	10.2	3.2	929.3	4.7	542	2.2
12.69	Luteolin-7-O-rutenoside	78.1	8.6	1.0	343.9	5.3	239.7	1.5
13.29	Diosmetin-8-C-glucoside	64.3	2.0	0.7	67.9	6.2	100.3	1.6
13.49	Diosmetin-6-C-glucoside	41.1	5.6	12.9	288.9	2.3	79.7	5.4
Total		352.2	32.3		2380.3		1259	
Flavanones								
10.64	Flavanone-derivate	134.6	8.9	2.4	619.7	4.3	183.8	2.7
12.26	Eriodictyol-7-O-rutinoside	1759.1	80.3	2.7	8498.4	5.2	5378	2.0
13.63	Naringenin-7-O-rutinoside	109.4	8.6	2.8	427.1	6.8	121.6	2.4
14.1	Naringenin-7-O-neohesperoside	58.1	30.3	5.9	117.6	9.1	57.1	1.9
14.59	Hesperetin-7-O-rutinoside	4144	488.9	2.8	23910.5	3.3	10423	1
Total		6205.2	617		33573.3		16163	

[a] Repeatability of the procedure for each sample type was determined by the analysis of one sample in triplicate.

3.2.2. Characterisation of primary and secondary metabolites in processed fruit fibres from the juice industry

CHARACTERISATION OF PRIMARY AND SECONDARY METABOLITES IN PROCESSED FRUIT FIBRES FROM THE JUICE INDUSTRY

Antoni Delpino-Rius [a], Alexis Marsol-Vall [a], Jordi Eras [a,b,*], Montse Llovera [b], Miguel Ángel Cubero [c,d], Mercè Balcells [a,*,] Ramon Canela-Garayoa [a]

[a] Chemistry Department, ETSEA, University of Lleida, 25198-Lleida, Spain

[b] Scientific Technical Service DATCEM, University of Lleida, 25198-Lleida, Spain

[c] Food Science and Technology Department, University of Lleida, 25198-Lleida, Spain

[d] Indulleida, S.A., N. 230, km 12, 25125-Alguaire, Lleida, Spain.

Submitted to *Food Composition and Analysis*

Keywords: Fruit fibres; primary metabolites; secondary metabolites; carotenoids.

ABSTRACT

Here we analysed the content of primary and secondary metabolites in nine types of industrially processed fibres derived from the juice industry. Specifically, we examined fibre from the following: apple, peach, and pear, as non-citrus fruits; the peel and flesh of orange and tangerine, and lemon flesh, as citrus fruits; and carrot, as vegetable. Regarding primary metabolites, the sugar content ranged from 21.6 mg/g in lemon to 290 mg/g in orange peel and lower mass organic acid content ranged from 25.0 mg/g in pear to 250 mg/g in lemon. The content and profile of fatty acids were constant during fibre processing, ranging from 0.5 to 1.46%. With respect to secondary metabolites, processing did not decrease the sterols, which ranged from 0.51 to 1.66 μg/g. Regarding carotenoids, of note was the presence of epoxycarotenoids, which may reflect the quality of the industrial process, thus giving added value to the by-product.

1. Introduction.

The juice industry produces large volumes of waste, both solid and liquid. Such waste is a source of pollution and also implies a loss of valuable biomass and nutrients [1]. This waste can be exploited to obtain fibres—these having distinct chemical compositions and physicochemical properties depending on the fruit from which they derive and its provenance. In this context, the effects of the physical, chemical, and functional properties of fibre from fruit and vegetable by-products on health have been reported. Greater knowledge of the chemical composition of fibres would provide insight into the physiological effects they generate and also contribute to the assessment of the loss of nutritional value after industrial processing.

The profile of primary metabolites is relevant in the chemical composition of fruit fibres. In some cases, the total percentage of fibre can be almost 36% of the weight of the product considered industrial fruit fibre. High sugar content and moderate concentrations of organic acids greatly contribute to the sensory attributes of fruit and dietary fibre from the same [2-4]. From the nutritional perspective, differences in carbohydrate will condition its glycaemic potential, moreover the content of certain organic acids in the fibre may lead to a lowering of postprandial blood glucose and insulin responses [5, 6]. Amino acids are also crucial for human nutrition and they affect the attributes of food, including taste, aroma, and colour [7]. Moreover, amino acids are useful markers to define fruit juice genuineness; however, their use is hampered by the natural variability of fruit composition. Despite the low concentration of lipophilic compounds in fruit, they play a key role in sensory quality. As unsaturated fatty acids predominate over saturated fatty acids in fruit and vegetables [8], the oxidation of the former during thermal treatment and storage may occur, resulting in the formation of compounds with an unpleasant aroma and flavour. However, the temperature applied to fibres during drying processes does not seem to affect their fatty acid content or profile [9].

According to the nomenclature adopted by the British Nutrition Foundation, plant secondary metabolites can be divided into four major groups, namely phenolic compounds, terpenoids, alkaloids, and sulphur-containing compounds [10]. Unlike primary metabolites, the so-called secondary metabolites are often unevenly distributed among taxonomic groups within the plant kingdom. Moreover, many of these metabolites have positive effects on human health. In a first approach to the chemical composition of fruit fibres, we previously reported on their polyphenolic profile and content [11]. Terpenoids are a family of chemical compounds derived from five-carbon isoprene units, and they play diverse functional roles. Examples of these compounds include sterols as structural components and carotenoids and phytols as photosynthetic pigments.

Plant sterols have known serum-cholesterol lowering effects [12]. Phytosterols have been widely studied in vegetables and fruits [13], but not in processed fibres from the same. Carotenoids are phytochemicals with anti-oxidant and health-promoting properties; they can reduce the risk of lifestyle-related diseases, including cancer [14]. Nevertheless, the presence of a long chain of conjugated carbon-carbon double bonds in carotenoids makes these compounds highly susceptible to degradation by heat, oxygen, light, and acids. During food processing and storage, carotenoids undergo a number of reactions, such as isomerisation (trans to cis), oxidation, and rearrangements of 5,6-epoxide to 5,8-epoxide in acidic conditions [15, 16]. While carotenoids have been reported in fruits and vegetables [17], little attention has been given to the characterisation of by-products and fibres obtained from these food matrices. Carotenoids—usually total content—have been addressed in carrot by-products [18], although orange dietary fibre[19] and by-products from tropical fruit [20] have also been studied.

Given that research into processed fruit fibres is unprecedented as only the anti-oxidant properties of these products have been reported previously [11, 20] and considering the interest of the fruit industry in the characterisation of its commercial products, here we selected nine fibres from industrial by-products obtained from the juice industry and analysed their composition. In this regard, we focused on the characterisation and quantification of primary metabolites (sugars, low molecular mass organic acids, amino acids, and fatty acids) and other secondary metabolites such as phytosterols and carotenoids in industrial fibres from various fruits and also carrots. UPLC-PDA-APCI/MS and GC-FID-MS methods were used to analyse the samples, which were prepared using polar and apolar solvents [21].

2. Material and methods.

2.1. Reagents, solvents, and standards.

Ethanol, 2-propanol, methanol (MeOH), ethyl acetate, acetonitrile (ACN), chloroform, and acetone (HPLC grade purity) were supplied by J.T. Baker (Deventer, The Netherlands). ACN and methanol (LC–MS grade) for chromatographic analysis were also from J.T. Baker. Water was obtained using a Milli-Q system from Millipore (Bedford, MA, USA). Sodium chloride and BHT (2,6-di-t-butyl-4-methylphenol) were provided by Fluka (Sigma-Aldrich, Madrid, Spain), potassium hydroxide by Panreac (Barcelona, Spain), and ascorbic acid by Acros (Pittsburgh, PA, USA). *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) and methoxyamine hydrochloride (MEOX) were purchased from Sigma-Aldrich (Madrid, Spain).

Succinic acid, quinic acid, shikimic acid, citric acid, fumaric acid, gluconic acid, citramalic acid, fructose, glucose, sorbitol, xylose, galactose, rhamnose, arabinose, proline, and phenylalanine were purchased from Sigma-Aldrich. Malic acid, myo-inositol, glycerol and a set of 21 L-amino acids plus glycine (ref LAA21) were supplied by Fluka. Phosphoric acid and lactic acid were supplied by Acros and Probus (Barcelona, Spain), respectively.

A set of 37 fatty acid methyl ester reference standards (10 mg/mL in dichloromethane) were purchased from Supelco (FAME MIX). Triundecanoin was used as internal standard and was supplied by Fluka.

β-Carotene, (all-E)-lutein, and β-cryptoxanthin were purchased from Sigma-Aldrich. (all-E)-Zeaxanthin was supplied by Fluka. Phytoene, (all-E)-violaxanthin, (9′Z)-neoxanthin, and (all-E)-antheraxanthin were provided by CaroteNature (Lupsingen, Switzerland). All stock standard solutions were prepared in ACN:MeOH:acetone (47:20:33, v/v/v) and stored at –80°C. Total carotenoid concentration was determined spectrophotometrically using a UV2 UV-vis spectrometer (ATI Unicam, Cambridge, UK). The described values of A1% 1 cm were used to quantify each carotenoid stock solution [22]. Working solutions were prepared from stock solutions by sampling an aliquot and diluting it with the injection solvent (ACN/MeOH/acetone, 47:20:33, v/v/v).

2.2. Samples.

The following batches of industrial fibre were provided by a local juice company (Indulleida, Alguaire (Lleida), Spain): 6 apple; 5 pear; 5 peach; 6 orange flesh; 5 lemon flesh; 1 orange peel; 1 tangerine flesh; 1 tangerine peel; and 1 carrot. Usually a batch is composed by fruits and vegetables of different varieties and geographical provenance. Fibre refers to the by-product obtained from the industrial processing of fruit and vegetable in the juice industry; a scheme of the process is shown in the Supplementary Data.

2.3. Determination of sugars, sugar alcohols, low molecular mass organic acids, and amino acids.

Extraction and derivatisation procedure.

Extraction and derivatisation followed a procedure similar to that described by [23]. Aliquots of 10 mg of fibre were placed in a 2.0-mL Eppendorf vial. Subsequently, 1.4 mL of a H_2O:MeOH (70:30 v/v) solution was added. The mixture was vortexed for 30 min and then centrifuged at 20,000 × g for 5 min. A volume of 150 µL of the extract was placed into a 1.5-mL Eppendorf vial. The extract was dried for 45 min at room temperature using a SpeedVac (Thermo, Asheville, NC, USA) apparatus. The residue was then suspended in 40 µL of a solution of MEOX in pyridine (20 mg/mL), vortexed until it was completely solved, and then incubated in a ThermoMixer (Eppendorf AG, Hamburg, Germany) for 1 h at 40ºC. Next, the vial was removed, and then 80 µL of MSTFA was added. The sample was vortexed and incubated at 40ºC for 1 h. Finally, the sample was placed in a chromatography vial containing a glass insert.

Gas chromatography analysis.

GC analyses were performed in a 6890N gas chromatograph equipped with a 5973 Mass Selective Detector, both from Agilent Technologies. The analytical column was a BPX-5 (SGE Analytical Science, Milton Keynes, UK) (95% dimethyl-, 5% diphenylpolysiloxane) capillary (30 m, 0.25 mm i.d., 0.25-µm film thickness). The temperature programme was 70ºC for 5 min, followed by an increase of 8ºC/min until reaching the final temperature of 360ºC, which was then held for 1 min. A 1:50 split injection ratio was applied. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The injection volume was 1 µL. The injection system was held at 280ºC.

Quantification was carried out by the external calibration curve method applying Single Ion Monitoring (SIM) by selecting two ions for each compound—one for chromatographic area quantification and the other one for confirmation purposes.

2.4. Fatty acid determination.

Transesterification of acylglycerides.

A volume of internal standard (50 µL) was added to a 10-mL reaction vial fitted with a poly(tetrafluoroethylene)(PTFE)-lined cap, and the solution was evaporated to dryness. Next, 350 mg of fibre, 2 mL of 2% sulphuric acid (H_2SO_4) in methanol with 2 mg of BHT were added, and the vials were vortex-mixed for 5 min. They were then filled with nitrogen and heated at 80ºC in a heating aluminium block with continuous magnetic stirring for 40 min. After cooling, 3 mL of a NaCl saturated aqueous solution and 2 mL of hexane were added. Vials were vortex-mixed (1 min) and centrifuged for 5 min at 433 x g. The upper phase was recovered. The aqueous phase was re-extracted with 2 mL of hexane, and the upper phases were combined.

Gas chromatography analysis.

Quantitative and qualitative analysis of FAMEs were carried out by GC with an Agilent 7890A apparatus coupled to an FID detector. The analytical column was a 30 m × 0.25 mm fused silica capillary coated with a 0.20-µm film of poly(80% biscyanopropyl-20% cyanopropylphenylsiloxane) (SP-2330; Supelco). The temperature programme was 100ºC for 1 min, followed by an increase of 8ºC /min until 200ºC and a second increase of 3ºC/min until reaching a final temperature of 225ºC, which was then held for 3 min. A 1:20 split injection ratio was applied in a split-splitless system. Helium was used as the carrier gas at a constant flow rate of 2 mL/min. The injection volume was 1 µL. The injection system was held at 250ºC and the FID system at 260ºC.

Additional qualitative analysis was performed using two systems, namely an Agilent 7890A gas chromatograph coupled to an FID detector, and an Agilent 6890N gas chromatograph coupled to a 5973 Mass Selective Detector. In both, the analytical column was a HP-5 (Supelco) (95% dimethyl-, 5% diphenylpolysiloxane) capillary (30 m, 0.25-mm i.d., 0.25-µm film thickness). The temperature programme was 100ºC for 1 min, followed by an increase of 5ºC/min until reaching the final temperature of 255ºC, which was then held for 3 min. A 1:20 split injection ratio was applied. Helium was used as the carrier gas at a constant flow rate of 2 mL/min. The injection volume was 1 µL. The injection system was held at 250ºC and the FID system at 280ºC.

Quantification was carried out by the internal standard method. The response factors of methyl esters of commercially available saturated and unsaturated fatty acids were calculated under the same chromatographic conditions as those indicated above. The repeatability of the method was assessed in each fibre by analysing one sample in triplicate.

2.5. Determination of phytosterols, triterpenes, fatty alcohols, and alkanes

Extraction and derivatisation procedure.

Extraction and derivatisation of free hydroxyl groups to trimethylsilyl ethers followed a procedure similar to that described by [24]. Aliquots of 10 mg of fibre were placed in a 10-mL glass vial with teflon cap. The fibre was then extracted with 0.8 mL of $CHCl_3$ for 12 h at 35 ºC, with shaking at 1400 rpm. Subsequently, 0.2 mL of a NaCl saturated aqueous solution was added. The mixture was vortexed and centrifuged at 20,000 × g for 5 min, and the organic layer was then recovered. The aqueous phase was re-extracted with 0.8 mL of $CHCl_3$. The organic extract was dried for 45 min at room temperature using a SpeedVac apparatus. The residue was then suspended in 200 µL of pyridine and 200 µL of BSTFA, vortexed until completely solved, and then incubated in a ThermoMixer for 15 min at 100ºC. Finally, the sample was placed in a chromatography vial containing a glass insert.

Gas chromatography analysis.

GC analyses were performed in a 6890N gas chromatograph equipped with a 5973 Mass Selective Detector, both from Agilent Technologies. The analytical column was a BPX-5 (95% dimethyl-, 5% diphenylpolysiloxane) capillary (30 m, 0.25 mm i.d., 0.25-µm film thickness). The temperature programme was 70ºC for 1 min, followed by an increase of 10ºC/min until reaching temperature of 100ºC, which was then held for 1 min. Finally, an increase of 5ºC/min was applied until a temperature of 310ºC was reached, which was then held for 10 min. A 1:5 split injection ratio was applied. Helium was used as the carrier gas at a constant flow rate of 2 mL/min. The injection volume was 1 µL. The injection system was held at 280ºC.

Quantification was carried out by the external calibration curve method and SIM by selecting two ions for each compound—one for chromatographic area quantification and the other for confirmation purposes.

2.6. Determination of carotenoids.

Extraction of carotenoids.

A method adapted from [21] was applied. Between 0.05 and 0.6 g of fibre was continuously shaken at 5000 rpm in a dark room with 3 mL of methanol in a 15-mL Falcon type tube (Multi Reax; Heidolph, Schwabach, Germany) for 10 min. Next, 6 mL of chloroform was added, and the vial was shaken again as before. Next, 2.3 mL of an aqueous solution of NaCl (10% w/v) was added, and the mixture was shaken for 15 min and cooled at 4ºC. The mixture was then centrifuged at 2,700 x g for 3 min at 4ºC, and the organic layer was recovered and dried under N_2. The residue was saponified for 60 min at 55ºC under N_2 in a shaking incubator 211DS Labnet (Edison, NJ, USA) using 1 mL of a solution of 6% KOH in methanol (w/v). After addition of 1 mL of sodium chloride solution (10%, w/v), the mixture was placed in a freezer for 15 min. Next, 2.5 mL of a hexane:diethyl ether solution (3:1, v/v) was added. The mixture was vortex-mixed and centrifuged at 433 x g for 3 min, and the organic layer was then recovered and reserved (this step was repeated until the aqueous phase was colourless). The organic layers were combined, and the solvent was removed under nitrogen stream. The residue was stored at –80 ºC under nitrogen until UPLC analysis. The dry residue was dissolved in the injection solvent immediately prior to the analysis of carotenoids.

Ultra-high-performance liquid chromatography parameters.

Chromatographic analysis was performed on a Waters ACQUITY UPLC™ system (Waters, Milford, MA, USA) consisting of ACQUITY UPLC™ binary solvent manager and ACQUITY UPLC™ sample manager, coupled to a photodiode array (PDA) 2996 detector. Compounds were separated with an ACQUITY UPLC™ BEH C18 column (1.7 µm; 1 mm × 100 mm) (Waters, Manchester, UK) using a gradient system consisting of solvent A, ACN–MeOH: (7:3, v/v), and solvent B, 100% H_2O. The linear gradient was as follows: 0–0.6 min, 25% B, 0.5 mL/min (isocratic); 0.6–6.5 min, 4.9% B, 0.5 mL/min (linear gradient); 6.5–7.5 min, 0% B, 0.7 mL/min (linear gradient); 7.5–13.6 min, 0% B, 0.7 mL/min (isocratic); 13.6–14.1 min, 25% B, 0.5 mL/min (linear gradient); and 14.1–16.6 min, 25% B, 0.5 mL/min (isocratic). Weak and strong needle solvents were ACN–MeOH (7:3, v/v) and 2-propanol, respectively. The injection volume was 5 µL, and the column was kept at 32ºC, while the temperature in the sample manager was maintained at 25ºC. The average maximum pressure in the chromatographic system was 62053 kPa (9000 psi). Instrument control and data acquisition and processing were carried out using MassLynx™ software (version 4.1; Waters, USA).

Repeatability of the procedure for each sample type was determined by the analysis of one sample in triplicate.

Mass spectrometry parameters.

MS analysis was carried out on a Waters ACQUITY TQD tandem quadrupole mass spectrometer (Waters, UK). The instrument was operated using an atmospheric pressure chemical ionisation (APCI) source in positive ion mode. The APCI parameters were as follows: corona voltage 4.0 kV; extractor voltage 3 V; source temperature 150ºC; probe temperature 450ºC; cone gas (nitrogen) flow 10 L/h; and desolvation gas (also nitrogen) flow 150 L/h. Collision-induced dissociation was achieved using argon at a flow rate of 0.15 mL/min in the collision cell. Data acquisition was performed using MassLynx 4.1 software (Waters).

3. Results and discussion.

3. 1. Primary metabolites. Sugars, sugar alcohols, low molecular mass organic acids, amino acids, and fatty acids.

Sugars.

In general, sugars were the most abundant group of compounds found by polar extraction of compounds followed by silylation, with the exception of lemon fibre, in which organic acids were the most abundant primary metabolites (Table 1). The sugar content of the nine fibres ranged from 21.6 mg/g in lemon to 290 mg/g in orange peel. Considering individual sugars, fructose, glucose, and sucrose accounted for over 90% of the sugar content in all fibres except those of lemon, in which they accounted for 82%; however, the ratio between sugars differed in the fibres. While lemon, apple, pear and orange peel presented higher contents of glucose and fructose, peach, orange flesh, tangerine and carrot registered higher values of sucrose. In contrast, galactose, rhamnose, raffinose, xylose and arabinose were found in minor concentrations. Comparing the sugar composition of apple fibre to the findings of other studies in which raw apples were analysed [25], we can affirm that sugar content was reduced 4-fold during fibre production, while the sugar profile was maintained. The same behaviour has been reported in peach [26], pear [27] and citric fruits [3]. However, variations in sugar composition are highly dependent on factors such as climate, the ratio of leaves to fruit, and the harvesting time. In addition, we found a higher content of sugars in the peel than in the flesh of orange, which is consistent with the literature [28].

Sugar alcohols.

The content of sugar alcohols ranged from 3.6 to 24.2 mg/g, lemon fibre presenting the lowest content (Table 1). This family of compounds comprised mainly sorbitol and myo-inositol, although a minor concentration of glycerol was also detected in some samples. Of note, the myo-inositol/sorbitol ratio was 5/27 for citrus samples while for apple, peach, and pear it was 3/27.

Low molecular mass organic acids.

The fibre samples presented between 25.0 and 250 mg/g of low molecular mass organic acids (Table 1). Lemon showed the highest content due to its elevated content of citric acid (225 mg/g), while the other fibres presented similar concentrations, ranging from 25.0 in pear to 60.6 mg/g in carrot. The distribution of individual acids in the different samples was variable; however, the main acids were malic, citric and quinic acid, the former being the major acid in all fibres except for lemon. In addition, succinic and lactic acid were, together with the organic acids indicated previously, the only acids found in all the samples. Moreover, we also report the content of phosphoric acid. Similar to sugars, the organic acid content of the fibres was lower than that of raw fruits, although the profile was again maintained [3, 26, 27].

Amino acids.

This was the group of compounds with the lowest content in the fibres, the total ranging from 1.2 mg/g for pear to 12.5 mg/g for carrot (Table 1). Alanine, glutamine, aspartic acid, and glycine were the only amino acids found in all the samples.

Table 1. Primary metabolites (sugars, sugar alcohols, low molecular mass organic acids and amino acids) content in fruit and carrot fibre.

Metabolite	**Apple**		**Pear**		**Peach**		**Orange flesh**	
	mg/g	RSD	mg/g	RSD	mg/g	RSD	mg/g	RSD
Fructose	116±26	5.4	81.6±18.8	3.1	55.6±26.5	3.6	66.6±6.1	2.5
Glucose	60.9±16.1	3.7	40.3±9.5	4.2	42±5.9	2.1	45.3±15.5	4.3
Galactose					2.0±5.0	7.1	7.1±5.5	6.4
Sucrose	38.1±17.4	5.1	16.4±7.8	4.2	114±21	6.7	99.8±27.6	3.2
Xylose	1.8±0.3	7.9	0.8±0.1	8.1	0.8±0.1	9.3	0.3±0.3	8.7
Arabinose	1.4±0.4	10.1	1.8±0.4	3.3	1.2±0.4	8.4	1.7±0.4	8.1
Total sugars	**224±60**		**141±24**		**216±29**		**221±45**	
Sorbitol	9.1±3.2	3.6	23.4±5.1	3.9	8.3±4.1	3.8	0.5±0.1	10.9
myo-Inositol	1.1±0.3	5.1	0.9±0.1	4.2	5.2±5.4	2.3	14.1±5.1	3.8
Total sugar alcohols	**10.7±3.3**		**24.2±5.1**		**13.6±2**		**14.7±5.2**	
Succinic acid	0.4±0.1	10.3	0.4±0.1	9.6	0.5±0	10.2	0.6±0.1	10.1
Malic acid	5.3±2.1	3.2	2.7±0.5	2.5	9±3.6	3.5	2.7±0.6	2.0
Citric acid	3.6±0.2	4.5	4.3±0.4	5.2	6.1±0.5	4.6	8.3±3.5	6.4
Quinic acid	14.8±0.9	3.2	15.9±1.6	2.7	38.5±12.3	8.5	14.8±1	3.4
Gluconic acid	2.4±4.6	5.6			1.6±1.7	7.8	2.3±1.8	7.9
Ferulic acid					0.2±0.4		1.1±0.1	
Phosphoric acid	0.2±0.2	12.4	0.1±0.1	7.9	0.6±0.2	8.9	1.5±0.3	9.8
Lactic acid	1.2±0.5	6.7	1.2±1	7.6	1.3±1	8.2	6.3±7.8	7.2
Total organic acids	**28.7±5.7**		**25.0±1.7**		**57.7±13.7**		**37.6±5.6**	
Alanine	0.1±0.1	7.8	0.1±0.1	9.6	0.2±0	9.2	0.2±0	8.7
Valine					0.3±0.4	8.7	0.3±0.4	6.9
Glutamine	0.5±0.3	11.2	0.3±0.3	9.8	0.8±0.1	9	0.8±0.1	11.5
Aspartic acid	0.7±0	12.4	0.7±0.1	13.3	0.8±0	11.8	0.8±0	9.5
Serine					1.6±0.8	9.2		
Proline			0.1±0	11.2	0.2±0.2	13.4	0.7±0.2	11.2
Total amino acids	**1.4±0.2**		**1.2±0.4**		**4.3±1.3**		**3.1±0.6**	

Table 1. Cont.

Metabolite	**Lemon flesh**		**Orange peel**		**Tang. flesh**		**Tang. peel**		**Carrot flesh**	
	mg/g	RSD	mg/g	RSD	mg/g	RSD	mg/g	RSD	mg/g	RSD
Fructose	6.5±1.3	4.5	94.0	4.2	24.6	3.4	89.7	4.5	67.0	3.4
Glucose	8.3±2.3	6.7	108	3.1	22.7	4.7	80.2	2.4	29.6	2.7
Galactose			18.7	3.2	5.3	5.6	17.4	5.6		
Sucrose	3.0±0.2	7.2	66.3	3.2	35.8	5.4	94.1	2.3	145	3.2
Xylose	2.6±0.3	6.5	0.8	12.2			1.2	10.9	1.1	7.6
Arabinose	0.5±0.1	9.1	2.5	10.1	0.5	10.3	2.0	9.8	1.4	5.9
Total sugars	**21.6±3.5**		**290**		**89.1**		**284**		**244**	
Sorbitol	0.5±0	11.2	0.5	7.6	0.4	6.9	0.6	10.1	3.1	5.3
myo-Inositol	2.8±0.3	3.6	11.6	8.4	4.0	7.4	15.5	4.2	5.6	2.9
Total sugar alcohols	**3.6±0.3**		**12.1**		**4.7**		**16.5**		**8.7**	
Succinic acid	0.6±0.3	9.5	0.4	8.9	0.5	7.8	0.5	11.2	3.1	6.3
Malic acid	5.3±0.8	4.3	1.7	5.6	2.0	3.6	1.8	3.0	5.6	2.5
Citric acid	225±26	2.3	8.5	3.1	7.2	2.7	13.2	3.7	3.9	5.3
Quinic acid	16.8±0.4	4.2	19.5	4.2	12.3	6.7	24.3	4.5	23.8	3.6
Gluconic acid			13.7	3.2			9.7	2.1	3.2	6.5
Ferulic acid			1.4				1.7			
Phosphoric acid	1.0±0.1	6.7	1.2	10.2	0.1	10.7	0.8	7.9	6.9	9.1
Lactic acid	1.2±0.3	10.7	1.7	11.2	1.6	12.3	2.6	8.3	13.7	5.9
Total organic acids	**250±27**		**48.1**		**24.6**		**54.7**		**60.7**	
Alanine	0.4±0	10.7	0.2	13.4	0.2	12.5	0.2	9.6		
Valine	0.8±0	7.6			0.6	9.8	0.8	11.0		
Glutamine	1.0±0.1	5.9	0.7	7.4	0.8	6.8	0.7	8.2	11.7	6.5
Aspartic acid	3.5±0.3	7.9	0.8	12.3	0.6	10.4	0.9	11.9	0.7	10.9
Serine	1.7±0.1	10.9			1.4	11.2				
Proline	1±0.2	11.7	0.6	12.1	0.2	12.4	1.0	10.5		
Total amino acids	**9.8±0.7**		**2.5**		**4.7**		**4.1**		**12.5**	

Mean and SD corresponding to n batches from different origin (n=6 apple fibres, n=5 pear fibres, n=5 peach fibres, n=6 orange flesh fibres, n=5 lemon flesh fibres, n=1 orange peel fibre, n=1 tangerine flesh fibres, n=1 tangerine peel fibre and n=1 carrot fibre). RSD of analytical method, expressed in %, corresponding to n=3 samples.

Fatty acids.

Table 2 shows the total fatty acid content and the saturated fatty acids (SFAs): monounsaturated fatty acids (MUFAs): polyunsaturated fatty acids (PUFAs) ratio for the fibres. Figure 1 shows the individual fatty acid distribution for each type of fibre.

Fatty acids were found in all the fibres, with concentrations ranging from 4.73 to 14.56 mg/g. Tangerine flesh exhibited the highest content (1.47 %), followed by tangerine peel (1.40%), orange flesh (1.10%), and orange peel (1.00%) fibres (Table 2). Thus the fatty acid content of fibre from flesh was slightly higher than the corresponding peel fibre. These results could be attributed to the presence of seed waste in flesh fibre. In contrast, peach and pear showed the lowest fatty acid content, with values close to 0.5%.

Of note, no great differences in total fatty acid content among batches were observed. Neither were differences centred on a particular compound. This finding is consistent with previous studies that described the variation of fatty acid concentrations and profiles in various cultivars of pear and orange, observing few differences among them [27, 29].

The flesh and peel of orange and tangerine showed a similar fatty acid profile. The percentage of SFAs was around 27%, while 73% of unsaturated fatty acids were distributed between ca. 25% of MUFAs and 45% of PUFAs. Palmitic acid (C16:0) was the main saturated fatty acid in the SFA profile. Similar percentages of oleic (9c-C18:1) acid and cis-vaccenic acid (11c-C18:1) were found in citrus fibre. The latter acid was detected only in citrus fibre. Linoleic acid (9c, 12c-C18:2 (n-6)) prevailed over α-linolenic acid (9c, 12c, 15c-C18:3 (n-3)) in the PUFA group, particularly in tangerine flesh fibre. The percentage of unsaturated fatty acids in orange flesh fibre (74%) was equivalent to that found in the juice of blood oranges (Arena et al.) The percentage of MUFAs and PUFAs in fibre from lemon flesh was 12 and 55%, respectively.

Table 2. Fatty acid content relative percentage of saturated and unsaturated fatty acids in fruit and carrot fibre.

Fibre	Fatty acid (%)[a]	RSD	Fatty acid composition (%) (SFA/MUFA/PUFA) [b]
Apple (n=6)	0.92 ± 0.19	3.9	32/18/50
Peach (n=5)	0.47 ± 0.10	4.5	44/16/39
Pear (n=5)	0.49 ± 0.06	4.9	35/26/39
Carrot (n=1)	0.75	6.7	37/7/59
Orange flesh (n=6)	1.10 ± 0.21	3.2	26/32/42
Lemon flesh (n=5)	0.80 ± 0.12	2.1	34/12/55
Tangerine flesh (n=1)	1.46	2.9	24/27/49
Orange peel (n=1)	1.00	3.5	31/23/46
Tangerine peel (n=1)	1.40	4.8	28/23/49

[a] Mean and SD corresponding to n batches.

[b] Percentage of total fatty acid content, namely saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs).

RSD of analytical method, expressed in %, corresponding to n=3 samples.

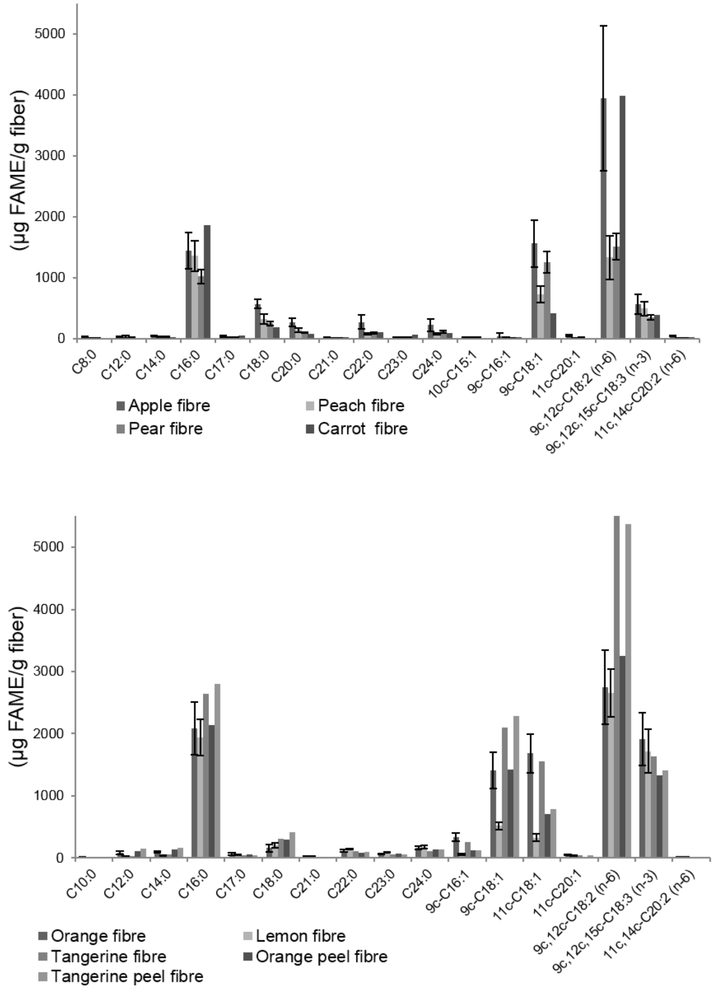


Figure 1. Fatty acid profiles of analysed fibres. Deviations corresponding to n batches n=6 apple, n=5 pear, n=5 peach, n=1 carrot, n=6 orange, n=1 tangerine, n=1 tangerine peel, n=5 lemon and n=1 orange peel.

The percentage of PUFAs in peach and pear fibre was 39% of the total fatty acids, where linoleic acid predominated over α-linolenic acid. In contrast, the percentage of SFAs and MUFAs differed between the two types of fibre. Oleic acid was the most abundant MUFA, although it was lower in peach than in pear fibre. Regarding SFAs, palmitic acid was more abundant than stearic acid (C18:0) and arachidic acid (C20:0). The percentage of unsaturated fatty acids in pear fibre (65%)

was slightly lower than that previously reported for pear (69 to 77%) [27]. In peach, the percentage of unsaturated fatty acids can range from 60 to 80%, depending on the stage of maturity and variety [8]. However, we found a slightly lower percentage of these fatty acids in peach fibre (56%). Apple fibre held 0.92% of fatty acids, of which 68% comprised unsaturated fatty acids, with 18% of MUFAs and 50% of PUFAs. In the PUFA profile, linoleic acid was clearly higher than α-linolenic acid, while for MUFAs oleic was predominant. Regarding SFAs, palmitic acid was the most abundant, followed by stearic, arachidic and behenic (C22:0) acids. Carrot fibre contained 0.75% of fatty acids, the profile of which showed the lowest percentage of MUFAs (7%) and the highest of PUFAs (59%). Linoleic acid was the main fatty acid in carrot, accounting for 53.4% of the total fatty acids. Palmitic acid was the next main fatty acid, accounting for 24.9%, followed by oleic and α-linolenic acids, with percentages around 5%.

3.2. Secondary metabolites. Phytosterols, triterpenes, fatty alcohols, alkanes, and carotenoids.

Phytosterols and triterpenes.

Total plant sterols ranged from 0.51 to 1.66 μg/g in the nine types of fibre studied (Table 3). The highest content was in orange fibre (1.66 μg/g) and the lowest in pear fibre (0.51 μg/g). β-Sitosterol and stigmasterol were found in all fibres, the former being the major phytosterol (0.41 to 1.01 μg/g) in all cases. Campesterol was present only in citrus fibres (Table 3). A comparison of profiles and contents with the findings of previous studies reveals that the industrial processing of fruits did not significantly affect the sterol composition of the resulting fibres. This observation is supported by the fact that cooking does not affect sterol content when compared with the content of raw samples [12]. Triterpenes were found only in non-citrus fibre and in carrot fibre. In these fibres, the content of triterpene was higher than that of phytosterol. Ursolic acid, oleanolic acid, and α-amyrin were identified. The maximum contents were found in apple fibre, with 42.83 μg/g, followed by pear and peach fibre, with 8.56 and 6.53 μg/g, respectively. In addition, the predominant triterpene was ursolic acid, followed by oleanolic acid and α-amyrin. The profiles and contents of these three compounds are similar to those reported previously in fresh fruit [30-32] and consistent with the findings of Geana et al. (2014), who described apple pomace as a suitable source of bioactive compounds such as ursolic and oleanolic acids [33].

Table 3. Triterpenes, phytosterols, fatty alcohols and alkanes content in fruit and carrot fibre.

Compound	Apple		Pear		Peach		Orange flesh		Lemon flesh		Orange peel	
	μg/g	RSD	μg/g	RSD	μg/g	RSD	μg/g	RSD	μg/g	RSD	μg/g	RSD
Campesterol							0.42±0.1	3.5	0.47±0.11	6.7	0.21	8.0
Stigmasterol	0.23±0.05	2.9			0.3±0.03	2.9	0.23±0.02	3.2	0.23±0.03	7.1	0.18	7.9
β-Sitosterol	0.93±0.33	4.2	0.51±0.15	7.6	0.42±0.1	4.2	1.01±0.24	6.1	0.78±0.2	11.6	0.41	11.9
Total phytosterols	**1.15±0.38**		**0.51±0.15**		**0.71±0.13**		**1.66±0.37**		**1.48±0.34**		**0.79**	
α-Amyrin	0.18±0.1	9.4	0.08±0.01	8.9	0.08±0.01	9.4						
Oleanolic acid	7.75±4.67	5.5	0.11±0.01	10.5	1.39±0.07	5.5						
Ursolic acid	34.9±11.5	6.2	0.12±0.01	12.5	3.85±2.86	6.2						
Total triterpenes	**42.8±16.3**		**0.31±0.03**		**5.32±2.94**							
Doconasol (C22)	0.39±0.02	4.8	0.78±0.23	6.0								
Tetraconasol (C24)	1.27±0.81	6.4	0.39±0.09	4.0	0.26±0.02	11.9			0.24±0.02	8.17		
Hexaconasol (C26)	3.06±1.83	8.4	0.4±0.05	2.9	0.4±0.03	7.6			0.35±0.03	7.80		
Octaconasol (C28)	3.73±2.03	8.1	0.44±0.07	3.6	0.39±0.03	6.4						
Total fatty alcohols	**8.45±4.69**		**2.01±0.45**		**1.05±0.08**				**0.58±0.05**			
Tricosane (C23)					1.07±1.53	4.8	3.22±0.36	4.94	2.34±0.21	6.5	2.46	8.17
Pentacosane (C25)	0.15±0.03	6.8	2.78±0.28	0.6	1.37±2.06	6.0	3.74±0.44	4.75	3.41±0.23	4.7	2.79	7.80
Heptacosane (C27)	0.7±0.35	12.1	0.18±0.03	4.4	0.37±0.08	3.5	0.14±0.01	9.8	0.15±0.01	8.7	0.13	7.80
Ocatacosane (C28)	0.22±0.05	5.9	0.15±0.02	3.0								
Nonacosane (C29)	9.27±5.3	13.8	2.86±0.92	9.5	0.36±0.09	2.3			0.16±0.02	4.7	3.51	4.8
Total alkanes	**10.3±5.7**		**5.97±1.25**		**3.16±3.76**		**7.10±0.81**		**6.06±0.46**		**8.89**	

Table 3. Cont.

Compound	**Tangerine flesh**		**Tangerine peel**		**Carrot flesh**	
	µg/g	RSD	µg/g	µg/g	RSD	µg/g
Campesterol	0.29	4.7	0.24	0.29	4.7	0.24
Stigmasterol	0.20	3.5		0.20	3.5	
Total phytosterols	**1.15**		**0.65**		**0.69**	
Tricosane (C23)	2.70	5.4	2.42	2.70	5.4	2.42
Pentacosane (C25)	3.06	4.7	3.09	3.06	4.7	3.09
Heptacosane (C27)	0.13	11.3	0.13	0.13	11.3	0.13
Nonacosane (C29)	3.48	5.6	3.47	3.48	5.6	3.47
Total alkanes	**9.36**		**9.1**		**10.04**	

Mean and SD corresponding to n batches from different origin (n=6 apple fibres, n=5 pear fibres, n=5 peach fibres, n=6 orange flesh fibres, n=5 lemon flesh fibres, n=1 orange peel fibre, n=1 tangerine flesh fibres, n=1 tangerine peel fibre and n=1 carrot fibre).
RSD of analytical method, expressed in %, corresponding to n=3 samples.

Fatty alcohols and alkanes.

In addition to the compounds described above resulting from apolar extraction, we also detected fatty alcohols and alkanes in this fraction (Table 3). While alkanes did not require derivatisation, fatty alcohols did. In fruit, fatty alcohols and alkanes have been found in the wax, which occurs primarily in the cuticle. The fatty alcohols identified corresponded to 22, 24, 26 and 28 carbon atoms, with apple fibre showing the highest value with an average of 8.45 µg/g. Of the citrus fibres, only lemon presented fatty alcohols. In contrast, alkanes were identified in all the samples, and the content did not vary greatly among them, ranging between 3.16 µg/g and 10.3 µg/g. The alkanes found in the different types of fibre corresponded to 23, 25, 27, 28 and 29 carbon atoms. The alkanes and fatty alcohols found were similar to those identified in fruit cuticle [24].

Carotenoids.

In terms of total carotenoid content, carrot fibre showed 22,250 µg/100 g in the single batch available (Table 4). This concentration is higher than those reported by Chantaro, Devahastin & Chiewchan (2008) in carrot peel fibre, which showed a decrease in β-carotene content as a result of thermal degradation during blanching and drying. We found that β-carotene (11,697 µg/100g) was the major carotenoid in carrot fibre, followed by high concentrations of α-carotene (6,871 µg/100g). Other carotenoids belonging to the carotene group, such as phytoene, phytofluene, and two isomers of β-carotene (A and B), were also detected, although in lower concentrations. With regard to the hydroxycarotenoid group, (all-E)-lutein was found (Table 4). The presence of these carotenoids has been reported in non-processed carrot [17].

Carotenoid compounds were not detected in lemon flesh fibre. After carrot fibre, the fibres of orange flesh and tangerine peel showed the highest content of total carotenoid (4,793 μg/100 g and 10,381 μg/100 g, respectively). The value for tangerine peel fibre is consistent with previous reports for total carotenoid content in tangerine peel flour (11,030 μg/100 g) [34]. In contrast, the total carotenoid content in orange peel fibre is higher than that previously described for orange peel flour (2,250 μg/100 g)[34]. Peel fibre had a higher concentration of carotenoids than flesh fibre (Table 4). Hence, the concentration of carotenoids in orange peel fibre was 2-fold and tangerine 10-fold that of flesh fibre. These findings are consistent with previous results corresponding to orange fibre [19] and citrus fruits [35]. The major carotenoids in orange and tangerine fruits are violaxanthin isomers, (all-*E*)-lutein, and (all-*E*)-β-cryptoxanthin [15]. In the case of citrus fibre, we found high contents of (all-*E*)-β-cryptoxanthin and phytoene, ranging from 394.9 to 774.0 μg/100g and 297.4 to 1,185 μg/100g, respectively. Phytofluene, (all-*E*)-lutein, (all-*E*)-zeaxanthin, α-carotene, and two isomers of β-carotene (A and B) were present at lower concentrations. α-Carotene was particularly abundant in tangerine peel fibre (1,764.0 μg/100g), thus supporting the notion that this compound is present mainly in peel [36]. The content and profile of carotenoids varied greatly in the six batches of orange flesh fibre. In this regard, (all-*E*)-β-Cryptoxanthin, β-carotene, and the epoxycarotenoids auroxanthin, neochrome and mutatoxanthin showed the highest variations in content (around 75%). In contrast, (all-*E*)-lutein, (all-*E*)-zeaxanthin, phytoene, and phytofluene showed slight variations (around 20%). It should be noted that the profile and concentrations of carotenoids depend greatly on the variety of the orange, the harvesting origin, and climatic factors [37].

Table 4. Carotenoid content of fibre.

Carotenoid	Peach		Orange flesh		Orange peel		Tangerine flesh		Tangerine peel		Carrot	
	μg/100g	RSD	μg/100g	RSD	μg/100g	RSD	μg/100g	RSD	μg/100g	RSD	μg/100g	RSD
Neochrome A	34.8±9.3	0.3		6.8	127	13	87.6	2.7				
Neochrome B	24.4±2.7	2.2		7.9		11						
Auroxanthin A	50.0±23.1	3.6	79.7 ± 74.2	4.5	558	11			1,454	3.4		
Auroxanthin B	30.9±12.2	8.4	44.1 ± 33.1		217	6.0			517	2.2		
Mutatoxanthin A	33.3±12.8	1.7	62.0 ± 41.7		238		56.9	1.7	793	3.2		
Auroxanthin C	39.9±22.9	2.7	88.4 ± 87.3	4.1	697	4.4			1813	4.2		
Mutatoxanthin B	31.2±10.2	1.1	68.1 ± 46.5	2.8	285	1.7	59.6	2.9	1,036	1.5		
(all-*E*)-Zeaxanthin	81.8±37.5	8.5	64.3 ± 12.2	2.9	143	8.8	83.7	4.9	638	5.9		
(all-*E*)-Lutein	128±30	5.6	66.4 ± 7.1	0.5	166	7.3	87.6	5.1	465	3.5	547	0.8
(all-*E*)-β-Cryptoxanthin	203±70	3.8	394 ± 314	2.8	462	10	513	9.5	774	12		
ζ-Carotene A	18.6±2.3	4.8	112 ± 56	4.8	429	7.7	32.6	12	775	10	659	2.5
ζ-Carotene B	16.2±3.0	2.1	112 ± 98	4.7	329	12	25.5	7	989	13	1378	5.7
α-Carotene							54.9	2.9			6,871	0.5
β-Carotene	180±68	5.3	32.2 ± 15.6	10	60.5	7.3	90.6	14	852	11	11,697	2.8
Phytofluene			62.3 ± 13.6	4.1	156	9.1	49	4.1	279	0.7	274	6.4
Phytoene	89.8±18.4	4.4	461 ± 120	5.5	926	6.7	297	2	1,185	3.7	819	3.6
Total carotenoids	**962 ± 322**		**1,648 ± 919**		**4,792.80**		**1,405**		**10,381**		**22,245**	

Table 4. Cont

Carotenoid	**Apple**		**Pear**	
	μg/100g	RSD	μg/100g	RSD
(all-*E*)-Lutein	74.4±24.7	8.6	74.4±24.7	8.6
(all-*E*)-β-Cryptoxanthin	23.3±3.2	3.5	23.3±3.2	3.5
β-Carotene	43.8±24.8	8.1	43.8±24.8	8.1
Phytoene	15.5±3.3	3.5	15.5±3.3	3.5
Total carotenoids	**157 ± 56**		**133 ± 19**	

As it is not possible to assign the chiral configuration of the 8 or 8′ carbon in these peaks using UV-vis absorption spectra and MS, they have been indicated as A, B and C.
Mean and SD corresponding to n batches (n=6 apple fibre, n=5 pear fibre, n=5 peach fibre, n=6 orange flesh fibre, n=1 orange peel fibre, n=1 tangerine flesh fibre, n=1 tangerine peel fibre and n=1 carrot fibre)
RSD of analytical method, expressed in %, corresponding to n=3 samples.

Special attention should be paid to the stability of some epoxycarotenoids during industrial processing, which usually leads to a decrease in the removal of violaxanthin, neoxanthin and antheraxanthin isomers [38]. In this regard, the organic acids released during the industrial processing of fruit juices decrease the pH enough to promote rearrangements of 5,6-epoxide to 5,8-epoxide [16]. In this context, we can explain the presence of auroxanthin (A, B and C) and mutatoxanthin (A and B) isomers, which are the epoxide–furanoid rearrangement products of violaxanthin and antheraxanthin, respectively [39] The concentrations of auroxanthin (212.2 μg/100 g) and mutatoxanthin (130.1 μg/100 g) in orange flesh fibre were similar to those of the most abundant carotenoids described above, while that of mutatoxanthin and neochrome A in tangerine flesh fibre was 116.4 μg/100 g and 54.2 μg/100 g, respectively. However, we would like to point out that the concentrations of these compounds were highly variable among the six batches of orange flesh fibre (around 80%). Auroxanthin and mutatoxanthin concentrations in citrus peel fibre were higher than in flesh fibre, ranging from 1,472 to 3,784 μg/100g and from 522.5 to 1,829 μg/100g, respectively. The differences in epoxycarotenoid concentrations between peel and flesh fibre were higher than those found for the other carotenoids.

We also found measurable amounts of carotenoids in peach and apple fibre. Regarding the total carotenoid content, peach fibre showed values close to 962.1 μg/100g. In contrast, the apple values were around 157.1 μg/100g. The content of carotenoids in these fruit fibres or similar products has not been described previously.

In peach fibre the major carotenoids were (all-*E*)-β-cryptoxanthin and β-carotene, followed by (all-*E*)-lutein, (all-*E*)-zeaxanthin, and phytoene. The epoxycarotenoids auroxanthin (120.8 μg/100g), neochrome (52.9 μg/100g), and mutatoxanthin (64.5 μg/100g) isomers were also detected. The carotenoid profiles were similar to those in commercial peach purees previously reported by our group [21]. The variability in the content and profile of carotenoids between the five batches of peach fibre was very similar to that shown by orange flesh fibre.

In contrast, the major carotenoid in apple and pear fibre was (all-*E*)-lutein (74.4 μg/100g and 50.2 μg/100g in apple and pear fibres, respectively). β-Carotene, (all-*E*)-β-cryptoxanthin, and phytoene were also present in these fibres.

In general, the carotenoid content in the fibres is consistent with that described for non-industrially processed products, and the width of carotenoid range is comparable with that of other processed products such as juices, purees, and jams. This study is the first to report on epoxycarotenoids in a by-product obtained from fruit. The content of these compounds, which are labile in acid media and high temperature, may provide a measure of the quality of the industrial processing of the fibres, keeping the valuable anti-oxidant compounds [18].

4. Conclusions.

Here we characterised the primary and secondary metabolites of nine batches of processed fibres from the juice industry—namely apple, peach, pear, orange peel and flesh, tangerine peel and flesh, lemon flesh, and carrot. Regarding primary metabolites, we observed that, after the juicing process, the fibres maintained significant amounts of sugars and organic acids. In general, the industrial process did not greatly affect the original profile of metabolites present Thus, the profile of sugars and organic acids in the fibres resembled that of the fresh fruit. Furthermore, the fatty acid profiles were unchanged in the fibres after processing. Moreover, the fatty acid content of the fibres ranged from 0.5 to 1.46%, thus indicating that these compounds are present in amounts sufficient to influence the organoleptic properties of these by-products.

Comparing the profiles and contents of the six tested fibres with the findings of previous studies on fresh fruits, we found that the processing of fruit to prepare juice and purees does not significantly affect secondary metabolites or minor compounds such as sterols or alkanes in the resulting fibres [12, 31]. Of note was the content of bioactive compounds, such as ursolic and oleanolic acids, in apple fibre. Among the nine industrially processed fibres addressed, carrot fibre showed the highest content of carotenoids, followed by orange and tangerine fibre. The carotenoid profile of the fibres was found to be similar to that of the corresponding fruit and vegetable. This is the first study to determine the epoxycarotenoids resulting from the rearrangements of 5,6-epoxide to 5,8-epoxide in fibres generated in the juice industry. The presence of epoxycarotenoids may reflect the quality of the industrial processing of the fibres, thus giving added value to the by-product.

Acknowledgement.

The authors would like to thank Indulleida S.A. (Lleida, Spain) for providing the samples.

References.

[1] G. Laufenberg, B. Kunz, M. Nystroem, Bioresource Technology, 87 (2003) 167.

[2] N. Grigelmo-Miguel, O. Martín-Belloso, LWT-Food Science and Technology, 32 (1999) 503.

[3] M.-V. Albertini, E. Carcouet, O. Pailly, C. Gambotti, F. Luro, L. Berti, Journal of agricultural and food chemistry, 54 (2006) 8335.

[4] A. Liverani, A. Cangini, Advances in Horticultural Science, 5 (1991) 59.

[5] K. Gustafsson, N. Asp, B. Hagander, M. Nyman, European journal of clinical nutrition, 48 (1994) 386.

[6] M. Suni, M. Nyman, N.A. Eriksson, L. Björk, I. Björck, Journal of the Science of Food and Agriculture, 80 (2000) 1538.

[7] J. Kirimura, A. Shimizu, A. Kimizuka, T. Ninomiya, N. Katsuya, J. Agric. Food Chem, 17 (1969) 689.

[8] Y. Duan, X. Dong, B. Liu, P. Li, Acta Physiol Plant, 35 (2013) 707.

[9] M.C.M. Moya Moreno, D. Mendoza Olivares, F.J. Amézquita López, J.V. Gimeno Adelantado, F. Bosch Reig, Talanta, 50 (1999) 269.

[10] G. Goldberg, Blackwell Publishing: Oxford, U.K.,, (2003).

[11] A. Delpino-Rius, J. Eras, F. Vilaró, M.Á. Cubero, M. Balcells, R. Canela-Garayoa, Food Chemistry, (2014).

[12] L. Normen, S. Bryngelsson, M. Johnsson, P. Evheden, L. Ellegård, H. Brants, H. Andersson, P. Dutta, Journal of food composition and analysis, 15 (2002) 693.

[13] V. Piironen, J. Toivo, R. Puupponen-Pimiä, A.M. Lampi, Journal of the Science of Food and Agriculture, 83 (2003) 330.

[14] N.I. Krinsky, Free Radical Biology and Medicine, 7 (1989) 617.

[15] A.J. Meléndez-Martínez, I.M. Vicario, F.J. Heredia, Journal of Food Composition and Analysis, 20 (2007) 638.

[16] R. Aparicio-Ruiz, B. Gandul-Rojas, Journal of Agricultural and Food Chemistry, 60 (2012) 5180.

[17] J. Burns, P.D. Fraser, P.M. Bramley, Phytochemistry, 62 (2003) 939.

[18] B.H. Chen, Y.C. Tang, Journal of Agricultural and Food Chemistry, 46 (1998) 2312.

[19] T. de Moraes Crizel, A. Jablonski, A. de Oliveira Rios, R. Rech, S.H. Flôres, LWT - Food Science and Technology, 53 (2013) 9.

[20] L.M.R.d. Silva, E.A.T.d. Figueiredo, N.M.P.S. Ricardo, I.G.P. Vieira, R.W.d. Figueiredo, I.M. Brasil, C.L. Gomes, Food Chemistry, 143 (2014) 398.

[21] A. Delpino-Rius, J. Eras, A. Marsol-Vall, F. Vilaró, M. Balcells, R. Canela-Garayoa, J Chromatogr A, (2014).

[22] G. Britton, S. Liaaen-Jensen, H. Pfander, 2004.

[23] J. Lisec, N. Schauer, J. Kopka, L. Willmitzer, A.R. Fernie, Nature Protocols electronic edition, 1 (2006) 387.

[24] B. Belge, M. Llovera, E. Comabella, J. Graell, I. Lara, Journal of Agricultural and Food Chemistry, 62 (2014) 3488.

[25] M. Suni, M. Nyman, N.-A. Eriksson, L. Björk, I. Björck, Journal of the Science of Food and Agriculture, 80 (2000) 1538.

[26] M. Colaric, R. Veberic, F. Stampar, M. Hudina, Journal of the Science of Food and Agriculture, 85 (2005) 2611.

[27] J. Chen, Z. Wang, J. Wu, Q. Wang, X. Hu, Food Chemistry, 104 (2007) 268.

[28] M.C. Garau, S. Simal, C. Rosselló, A. Femenia, Food Chemistry, 104 (2007) 1014.

[29] E. Arena, S. Campisi, B. Fallico, E. Maccarone, Journal of Agricultural and Food Chemistry, 46 (1998) 4138.

[30] J. Zhang, Z.-Liu,, Acta Horticulturae Sinica, 5 (2009) 005.

[31] R.D. Belding, S.M. Blankenship, E. Young, R.B. Leidy, Journal of the american society for horticultural science, 123 (1998) 348.

[32] X. Li, T. Wang, B. Zhou, W. Gao, J. Cao, L. Huang, Food Chem, 152 (2014) 531.

[33] E.-I. Geană, R.E. Ionete, A. Ciocarlan, A. Aricu, A. Fulga, N. Ungur, M. Podogova, D. Nikolaeva, Progress of Cryogenics & Isotopes Separation, 17 (2014).

[34] A.M. Rincón, A. Vásquez, M. Padilla, F. C, Archivos Latinoamericanos de Nutrición, 55 (2005) 305.

[35] Y.-C. Wang, Y.-C. Chuang, H.-W. Hsu, Food Chemistry, 106 (2008) 277.

[36] R. Rouseff, L. Raley, H.-J. Hofsommer, Journal of Agricultural and Food Chemistry, 44 (1996) 2176.

[37] A.-L. Fanciullino, M. Cercós, C. Dhuique-Mayer, Y. Froelicher, M. Talón, P. Ollitrault, R. Morillon, Journal of Agricultural and Food Chemistry, 56 (2008) 3628.

[38] A.J. Meléndez-Martínez, I.M. Vicario, F.J. Heredia, Food Chemistry, 101 (2007) 177.

[39] L.Q. Zepka, A.Z. Mercadante, Food Chemistry, 117 (2009) 28.

3.3. Analysis of primary metabolites and phenolic compounds to authenticate monovarietal fruit juices.

Authentication of fruit juices is a challenge for an industry that is growing fast and expanding. This chapter focuses on two of the objectives of this thesis; the first one is the authentication of monovarietal fruit juices, the second one is to address the effect of fruit processing to obtain high quality monovarietal juices. In the first chapter, an approach was made to study the carotenoid content and profile of freshly made and commercial monovarietal juices. This study included two industrial monovarietal peach juices, Spring Lady and Miraflores, and also three industrial monovarietal pear juices, Alejandrina, Conference and Blanquilla, and their corresponding fresh fruits. To authenticate and characterise the fruits from different cultivars, several techniques have been applied [1]. The objective in this study was to compare and combine the results obtained using NMR (primary metabolites) and UPLC-PDA-MS (phenolic compounds) with the goal to ascertain whether these analytes allow the differentiation of peach puree and pear juices of different varietal cultivars while simultaneously assessing the possibility of applying the developed methodology to fresh fruit and industrially manufactured juices and purees.

Most of the studies to authenticate fruits are based on multivariate statistical analyses used to evaluate the information obtained from an analytical technique [2]. Chemometric tools have been used to determine the addition of foreign materials, to differentiate among different juice commodities or even to evaluate geographical origin of a juice [3, 4]. In this study, NMR spectroscopy and chromatographic data were evaluated by PCA in combination with suitable preprocessing. PCA allows to find combinations of variables that explain the variance present in a data set. For each principal component (PC), every sample has a score, and every variable has a loading that represents its contribution to the combination.

The results of this study have been submitted to *Food Control* and are part of the project "Study of a new cold extraction process for the production of fruit juices and purees and monovarietal authentication" (2009-2013).

References.

[1] M. Dennis, Springer Science & Business Media, 1998.

[2] M. Cuny, E. Vigneau, G. Le Gall, I. Colquhoun, M. Lees, D. Rutledge, Analytical and Bioanalytical Chemistry, 390 (2008) 419.

[3] N. Tzouros, I. Arvanitoyannis, Critical Reviews in Food Science and Nutrition, 41 (2001) 287.

[4] J. He, L.E. Rodriguez-Saona, M.M. Giusti, Journal of Agricultural and Food Chemistry, 55 (2007) 4443.

3.3.1 Combined analysis of primary metabolites and phenolic compounds to authenticate commercial monovarietal peach purees and pear juices.

COMBINED ANALYSIS OF PRIMARY METABOLITES AND PHENOLIC COMPOUNDS TO AUTHENTICATE COMMERCIAL MONOVARIETAL PEACH PUREES AND PEAR JUICES

Antoni Delpino-Rius,† Jordi Eras Joli,*†‡ Ferran Gatius Cortiella,† Mercè Balcells Fluvià,*† and Ramon Canela Garayoa†

†Department of Chemistry, University of Lleida-DBA R+D Tecnio Center, Avda. Alcalde Rovira Roure, 191, E-25198, Lleida, Spain.

‡Scientific Technical Service DATCEM, University of Lleida, Avda. Alcalde Rovira Roure, 191, E-25198, Lleida, Spain.

Submitted to *Food Control*

Keywords: Authentication, PCA, primary metabolites, phenolic compounds, NMR, UPLC-PDA-MS/MS

ABSTRACT

Here we authenticated single-varietal peach purees and pear juices on the basis of primary metabolite and phenolic compound analysis by 1H-NMR and UPLC-PDA-MS/MS, respectively. After suitable preprocessing, the 1H-NMR and chromatographic data were evaluated by PCA, both separately and pooled. The PCA of phenolic compounds allowed better cluster separation than of primary metabolites. The PCA of primary metabolites allowed the cluster separation of purees of distinct peach varieties but not between processed and non-processed purees. For pear juices, both PCA approaches allowed satisfactory discrimination of Alejandrina, Conference, and Blanquilla cultivars. The PCA of combined data of primary metabolites and phenolic compounds improved the separation of the clusters in all cases, permitting the discrimination of processed and non-processed peach purees. This approach may help better control of cultivar authenticity in fruit products. Consequently, it may contribute to the development of a process to achieve products marked by a characteristic quality of a given cultivar.

1. Introduction.

Peach and pear are two of the most widespread fruit crops in Southern Europe. Consequently, the production of processed products (fruit juice, jam, nectar…) is an important industrial activity in this region. Peach flavor (3.7%) ranks fourth in the juices and nectars most consumed in the EU, behind orange (38.5%), mixtures of flavors (19.9%), and apple (13.3%). Pear is included in the 21% of other flavors consumed, although it is noteworthy that in countries such as Italy pear accounts for market shares of up to 12.5% [1]. To attract consumers, producers tend to be highly innovative when offering new products. Indeed, they have recently developed single-varietal juices. Juices are valuable commodities then is necessary to control through the juice analysis the economic fraud, for example adding a co-fruit, a less expensive fruit or the juice of a fruit that is easier to find [2-4]. In this context, the production of monovarietal juices do further opportunities for fraud, while increasing difficulty in detection, then novel methods to control its authenticity are welcome and interesting for consumers . In this regard, knowledge of the chemical composition of each cultivar and the effect of blending can contribute to the production of processed products with a controlled quality.

To authenticate and characterize fruit cultivars, several techniques have been applied, such as liquid chromatography[5], gas chromatography[6], near infrared spectroscopy[7], nuclear magnetic resonance[3, 8, 9], and mass spectrometry[8]. These techniques allow the analysis of various metabolites, such as amino acids, organic acids, sugars, phenolic compounds, and carotenoids. NMR and LC-PDA-MS are especially suitable for the analysis of food and drink, since both have excellent reproducibility and generally have minimum sample preparation requirements. NMR spectroscopy has been used to analyze primary metabolites (amino acids, organic acids, and sugars), while LC-PDA-MS has been successfully applied for the analysis of phenolic compounds in fruit samples [10-12].

Several studies have addressed the characterization and classification of peach cultivars on the basis of their phenolic profile [13-15]. Also, NMR has been applied to compare two varieties of peach with distinct resistance to pests [16]. Moreover, pear cultivars have been described on the basis of their phenolic profile, mainly their flavonol glycoside composition [17-19]. For peach and pear juices, the effects of the different processing steps on compounds have also described [20-23].

Here we sought to compare and combine NMR for primary metabolites and UPLC-PDA-MS for phenolic compounds in order to ascertain whether these analytes allow the differentiation of the cultivars used for peach puree and pear juice. This approach could enhance the control of cultivar authenticity in fruit products. Hence, the method could contribute to the development of processes devoted to the preparation of products with a characteristic quality of a given cultivar.

2. Material and methods.

2.1 Chemicals.

Methanol (MeOH) and acetone (HPLC grade purity) were supplied by J.T. Baker (Deventer, The Netherlands). The chromatographic solvents were acetonitrile (ACN) (LC-MS grade), also from J.T. Baker, and water, purified in a Milli-Q system from Millipore (Bedford, MA, USA). The chromatographic eluent additive was acetic acid (HAc) (LC-MS grade), provided by Fluka (Sigma-Aldrich, Madrid, Spain), and formic acid (HFor) and hydrochloric acid 37% (analytical grade), supplied by Merck. Sodium hydroxide was from Panreac (Barcelona, Spain) and ascorbic acid from Acros (Pittsburgh, PA, USA). Citric acid, malic acid, quinic acid, fructose, glucose, sucrose, and amino acids were supplied by Fluka.

The phenolic standards (+)-catechin, (−)-epicatechin, procyanidin B1, procyanidin B2, arbutin, eriodictyol-7-*O*-rutinoside, naringenin-7-*O*-rutinoside, naringenin, hesperetin-7-*O*-rutinoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-rutinoside, kaempferol-3-*O*-rutinoside, and isorhamnetin-3-*O*-rutinoside were supplied by Extrasynthèse (Genay, France). Arbutin, gallic acid, phloretin-2'-*O*-β-glucoside, 5'-caffeoylquinic acid, caffeic acid, *p*-coumaric acid, ferulic acid, and sinapic acid were purchased from Sigma–Aldrich Chemie (Steinheim, Germany). All stock standard solutions of phenolic compounds were prepared in methanol and stored at –80°C. Working solutions were prepared from stock solutions by sampling an aliquot and diluting it with the injection solvent (0.1 % acetic acid aqueous solution).

2.2. Samples.

Six commercial peach purees (*Prunus persica*) of Spring Lady and Miraflores cultivars and their corresponding fresh fruit were provided by a local fruit juice company (Zucasa, Fraga, Spain). Peaches were stoned and four pieces were homogenized in a blender (Grindomix GM 200; Retsch, Haan, Germany) at 5000 rpm for 2 min. Ascorbic acid (ca. 10 g/kg) was added to prevent oxidation in this blending step. The same company provided six pear juices derived from Alejandrina, Conference, and Blanquilla cultivars.

The following codes were assigned to the peach puree samples: FB: Freshly blended; C: Commercial; SL: Spring Lady cultivar; and M: Miraflores cultivar. An ending number was added to indicate the batch number (from 1 to 6 peach purees).

The following codes were assigned to the pear juice samples from the distinct cultivars: AJ: Alejandrina juice; CJ: Conference juice; and BJ: Blanquilla juice. An ending number was added to indicate the batch number (from 1 to 6 pear juices).

2.3. Sample preparation.

Samples for the NMR analysis: 45 g of juice or puree was clarified by centrifugation (13500 x *g*, 30 min, Hettich EBA 21). Maleic acid was added as internal standard at a concentration of 15 g/L. The supernatant was adjusted to pH 6.5 by addition of NaOH (5 M, 1 M and 0.1 M). The samples were passed through a 0.22-µm PTFE filter prior to recording the spectrum.

Samples for the UPLC analysis: 2 g of juice or puree was centrifuged at 30,400 x *g* (Hettich Eppendorf Centrifuge MIKRO 22 R; Germany). 0.5 mL of the supernatant was diluted 1:2 with Milli-Q water (acidified at 0.1%, v/v with acetic acid) and passed through a 0.22-µm PTFE filter. Solutions were kept at 6ºC until UPLC analysis. All solvents contained ascorbic acid (0.2 % w/v).

2.4 ^{1}H NMR analysis.

A volume of 600 µL of the sample (from juice or puree samples) and 100 µL of D_2O were introduced into an NMR tube with an outer diameter of 5 mm. One-dimensional spectra were recorded on a Varian AS 400 spectrometer operating at 400 MHz. The following acquisition parameters were used: spectral width 6402 Hz; relaxation delay 15 s; number of scans 128; acquisition time 4.09 s; and pulse width 90°. Experiments were carried out at 25°C. The recycle delay between scans was set to $5T_1$ of the malic acid, the longest value of T_1. T_1 relaxation time was determined using the inversion recovery pulse sequence. This procedure ensured complete relaxation of the molecules present in the fruit juices and of the internal standard from the NMR signals. Spectra were Fourier-transformed and weighted with a Gaussian function. Baseline correction was applied across the whole spectral range.

2.5 UPLC analysis.

UPLC-PDA-FLR parameters.

Ultra performance liquid chromatographic analysis was performed on a Waters ACQUITY UPLC™ system (Waters, Milford, MA, USA) consisting of an ACQUITY UPLC™ binary solvent manager and ACQUITY UPLC™ sample manager, coupled to a photodiode array detector ACQUITY UPLC™ PDA and fluorescence detector ACQUITY UPLC™ FLR. Compounds were separated with an ACQUITY UPLC™ HSS T3 column (1.8 μm; 2.1 mm × 150 mm) (Waters, Manchester, UK) using a mobile phase consisting of solvent A, H_2O (0.1% v/v HAc), and solvent B, ACN 100% (0.1% v/v HAc). The flow rate was 0.55 mL/min. The linear gradient was as follows: 0–1.89 min, 1% B, (isocratic); 1.89–17.84 min, 30% B, (linear gradient); 17.84–21.39 min, 5% B, (linear gradient); 21.39–21.56 min, 1% B, (linear gradient); and 21.56–25 min, 1% B, (isocratic). Weak and strong needle solvents were H_2O (0.1% v/v HAc) and MeOH, respectively. The injection volume was 20 μL in full loop mode, and the temperature in the column was kept at 45ºC and that in the sample injector at 10ºC.

Tables were built by noting chromatographic peak areas of the following phenolic families measured at specific wavelengths: 320 nm for hydroxycinnamic acids; 283 nm for flavanones; 285 nm for hydroquinone; and 340 nm and 350 nm for flavonols (isorhamnetin, kaempferol and quercetin glycosides). Fluorescence values for flavan-3-ols were measured at 310 nm (λ_{ex}=280).

UPLC-MS/MS parameters.

MS analysis was carried out on a Waters ACQUITY TQD tandem quadrupole mass spectrometer (Waters, UK). The instrument was operated using an electrospray source (ESI) in positive and negative ion mode. The following ESI parameters were used: capillary voltages of 3.5 kV and -2.5 kV in positive and negative mode, respectively; the source at 150ºC; desolvation temperature at 500ºC; cone gas (nitrogen) flow of 50 L/h; and desolvation gas flow of 800 L/h. Flow injections of each individual standard were used to optimize the cone voltage and Multiple Reaction Monitoring (MRM) parameters. Collision-induced dissociation was achieved using argon at a flow rate of 0.15 mL/min in the collision cell. MS conditions of standard compounds with same aglycone were applied to identify compounds for which standards are not available. MassLynx 4.1 software (Waters, USA) was used for data acquisition.

2.6. Chemometric analysis.

Chemometric analysis was performed using Statgraphics Centurion XVI Version: 16.2.04 (StatPoint Technologies, Warrenton, Virginia, USA). Correlation analysis was carried out to determine the relationships between the main components. We used Principal Component Analysis (PCA) as the projection technique for the multivariate analysis of data.

3. Results and discussion.

3.1. ^{1}H NMR and chemometric analysis of primary metabolites.

^{1}H NMR analysis of the peach puree and pear juices detected primary metabolites. In this regard, sugars, organic acids and amino acids were the metabolites present in high concentrations in fruits. A pH of 6.5 was selected on the basis that it gave the best resolution among the signals of the main compounds along the ^{1}H spectrum. The signals of these compounds were identified in the ^{1}H spectrum by means of their diagnostic signals [16] and confirmed through spiking experiments with standards. The characteristic signals of alanine (ALA)(β-CH_3) and asparagine (ASP)(β, β'-CH_2) were also identified in the ^{1}H spectrum as free amino acids. Table 1 shows the chemical shifts of the signals of the metabolites selected. Maleic acid (2 x α-CH) at 6.2 ppm was used as internal standard. To assess the suitability of this acid as an internal standard for the compounds chosen, a pear juice sample was fortified with the sugars, organic acids, and amino acids at various concentrations. The behavior of all the compounds was linear over the range, thereby confirming that maleic acid at a concentration of 15 g/L is a suitable internal standard for fruit samples. The ratio between the metabolite signal area and signal area of maleic acid at 6.2 ppm was used to build the PCA model.

Table 1. Summary of the ^{1}H NMR signals for the spectra of pear and peach used for the statistical analyses.

Compound	Assignment	^{1}H (ppm Hz)
Alanine (ALA)	β-CH_3	1.49
Quinic acid (QA)	CH_2-1,1'; CH_2-5,5'	1.7
Citric acid (CA)	α,γ-CH	2.3
Asparagine (ASP)	β, β'-CH_2	2.7
β-Glucose (βGLC)	CH-2	2.95
β-D-Fructopyranose(FRU)	CH_2-6,6'	3.85
Malic acid (MA)	β, β'-CH_2	4.1
α-Glucose (αGLC)	CH-1	4.95
Sucrose (SUCR)	GLC CH-1	5.15
Maleic acid (IS)	2CH	5.9

The abbreviation for each compound is shown in brackets.

The PCA was applied to determine the main sources of variability present in data sets and to establish the relation between samples (objects) and primary metabolites (variables). The weight of variables was normalized by the ratio of their standard deviation in the data set. In both models, the first two PCs (PC1 and PC2) plotted were selected to provide the highest variation of data objects (PC1 and PC2 explained 43% and 33% of the information for peach purees and 60% and 24% for pear juices, respectively).

The scores of the data presented in the PCA bi-plot (Figure1) for peach purees showed a moderate separation through PC1 between the Miraflores (M) and Spring Lady (SL) cultivars in both commercial (C) and freshly blended (FB) purees. The loadings of the variables used in the model reveal that the separation was on the basis of citric acid (CA) and asparagine (ASP) against sucrose (SUCR), quinic acid (QA) and malic acid (MA) (Pearson correlation -0.68, $p<0.05$). The weight in Spring Lady (SL) of citric acid (CA)) and asparagine (ASP) were higher than in Miraflores (M). In contrast, sucrose (SUCR) and quinic acid (QA) weights were higher in Miraflores (M). PCA scores did not show a separation between commercial (C) and freshly blended (FB) peach purees of either cultivar. The information in the PCA model was consistent with previous results, which showed no effect of fruit processing on the primary metabolites detected by ^{1}H NMR in the juices.[24].

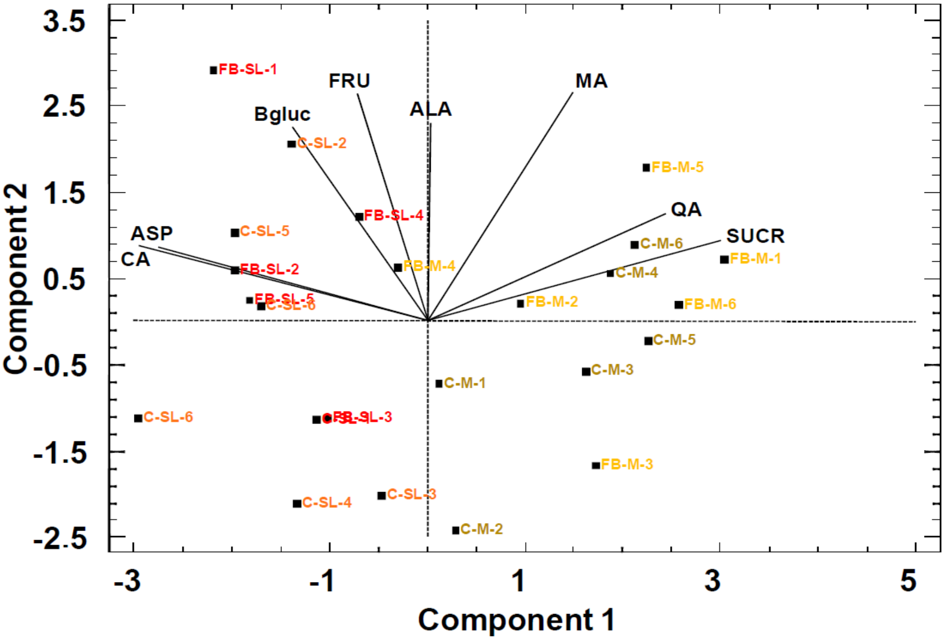


Figure 1. PCA bi-plot of peach purees with primary metabolites as statistical variables. FB: Freshly blended, C: Commercial, SL: Spring Lady cultivar, and M: Miraflores cultivar. Ending number is the batch number. PC1: 43%, PC2:33%.

The scores in the PCA bi-plot displayed results largely consistent with the origin of the pear cultivar, discriminating the 18 pear juice batches in three groups, namely Alejandrina (AJ), Blanquilla (BJ) and Conference (CJ) (Figure 2). The juice scores showed a clear separation in PC1 between Blanquilla (BJ) and Alejandrina (AJ) and Conference (CJ) and in PC2 between Alejandrina (AJ) and Blanquilla (BJ). The loadings of the variables in the plot show that the separation along PC1 is described by differences in acid content. In this case, the PC1 revealed a strong correlation of organic acids and amino acids (Pearson correlation between 0.70–0.92, $p<0.05$) and the samples with high acid content belonging to the Blanquilla (BJ) cultivar. In contrast, the separation on PC2 was based on the strong negative correlation between sucrose (SUCR) and glucose (αGLC and βGLC).

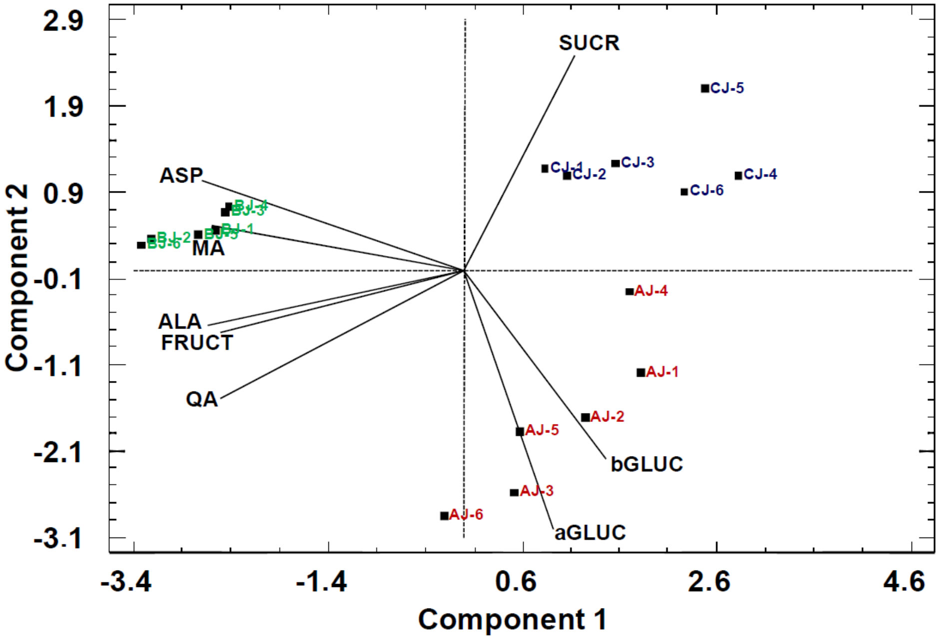


Figure 2. PCA bi-plot of pear juices with primary metabolites as statistical variables. AJ: Alejandrina juice, CJ: Conference juice, and BJ: Blanquilla juice. PC1: 60%, PC2: 24%.

3.2. Chemometric analysis of phenolic compounds determined by UPLC-PDA-FLR-MS/MS.

Table 2 summarizes the chromatographic, UV-vis, and MS parameters used for determination of the phenolic compounds in the samples. Identification of these compounds in fruit juices and purees was done on the basis of available standards and other tools that are useful to identify the maximum number of peaks, such as product ion mass spectrometry experiments and the chromatographic behavior and UV-Vis absorption spectra, together with published data on the main phenolic compounds in the fruit addressed, were used for identification purposes. We confirmed the structures of the phenolic compounds detected using the molecular ion and fragmentation pathways of MS. Caffeoylquinic and *p*-coumaroylquinic acids were identified in the derivatives of both fruits. The isomers 3, 4 and 5 of *p*-coumaroylquinic and caffeoylquinic acids were distinguished on the basis of the product ion spectra from $[M-H]^-$ and relative intensities of their ions. The characteristic ions were 191, 173 and 163 m/z in *p*-coumaroylquinic acid isomers and 191, 179 and 173 m/z in caffeoylquinic acid isomers[25]. Flavan-3-ols were identified from the standards and confirmed in the samples on the basis of their characteristic fragmentation ions. Fluorescence response allows a more selective and sensitive integration of compounds (λ_{ex}=280; λ_{em}=310 nm). Flavonoids are a widespread family of phenolic compounds, and we had access to at least one standard of each of the major families present in the fruits addressed. UV-Vis and mass spectra of these standards provided an effective tool to distinguish the aglycone family and allowed us to identify the flavonoids present. To tentatively identify the compounds for which standards were not available, we followed the practical guidelines for the characterization of glycosyl flavonoids by triple quadrupole MS[26, 27]. Also, studies reporting the flavonoid profiles of the fruits addressed here were useful [15, 17, 28, 29]. In peach purees, glycoside flavonols with the aglycones quercetin and kaempferol were identified, which have the characteristic fragments 303/301 for quercetin glycosides and 287/285 for kaempferol glycosides in positive and negative mode, respectively. Moreover, flavonol glycosides belonging to isorhamnetin aglycone were detected in pear juices, which presented the fragment 317/315 due to the loss of sugar in positive and negative mode, respectively.

Table 2. The chromatographic, UV-vis, and MS parameters of the phenolic compounds identified in the samples.

R.t. (min)	**Compound**	**UV max (nm)**	**$[M-H]^-$/$[M+H]^+$ (m/z)**	**Relative abundances of main ions detected from MS/MS of $[M-H]^-$/$[M+H]^+$**	**CE (eV)**
2.53	Hydroquinone β-D-glucopyranoside or Arbutin[a] (Arb)	283	271/273	-/108(100), 95 (18)	20
5.85	Protocatechuic aldehyde monoglucuronide [b] (ProCat-glu)	268	313/315	175/	20
6.45	3-Caffeoylquinic acid [b] (3-Caf-qui)	240, 326	353/355	-/191 (100), 179 (2), 171(1)	20
7.02	Coumaroyl-hexose [b] (Cou-hex)	316	325/327	163/	20
7.68	Procyanidin-B1 [a] (Pro-B1)	278	289/291	291(100), 409(88), 427(64), 247(64), 301(64) /407(100), 289(63), 425 (34)	20
7.96	5-Caffeoylquinic acid [a] (5-Caf-qui)	240, 326	353/356	163(100), 145(62), 135(20), 117(20)/191 (100), 179 (94)	20
8.22	(+)-Catechin [a] (Cat)	278	289/291	139(100), 123(47), 147(22)/203(100), 109(94), 125(84)	20

The abbreviation used for each compound is shown in brackets.

[a] Identification on the basis of the UV-vis spectrum and specific mass fragments of the standard compounds.

[b] Tentative identification on the basis of the UV-vis spectrum, MS, and HPLC/UHPLC retention times and published data.

Table 2. Cont

R.t. (min)	**Compound**	**UV max (nm)**	**$[M-H]^-$/$[M+H]^+$ (m/z)**	**Relative abundances of main ions detected from MS/MS of $[M-H]^-$/$[M+H]^+$**	**CE (eV)**
8.43	4-Caffeoylquinic acid [b] (4-Caf-qui)	240, 326	353/357	-/173 (100), 179(67),191(32)	20
9.23	Procyanidin-B2 [a] (Pro-B2)	278	577/579	291(100), 409(88), 427(64), 247(64), 301(64) /407(100), 289(63), 425 (34)	20
9.47	5-*p*-Coumaroylquinic acid [b] (5-p-Cou-qui)	235,312	337/339	-/191(100), 163(22)	20
9.88	(-)-Epicatechin [a] (Epi)	278	577/580	139(100), 123(47), 147(22)/203(100), 109(94), 125(84)	20
12.49	Quercetin-3-O-rutinoside [a] (Q-rut)	255, 353	609/611	303(100)/300(100)	30
12.59	Quercetin-3-O-galactoside [a] (Q-gal)	255, 353	463/465	303(100), 153(26)/300(100), 271(27), 255(19)	30
12.82	Quercetin-3-O-glucoside [a] (Q-glu)	255, 353	463/465	303(100), 153(26)/300(100), 271(27), 255(19)	30
13.64	Kaempferol 3-O-rutinoside [a] (K-rut)	265, 347	593/595	287/-	30
13.8	Isorhamnetin-3-O-robinioside [b] (Iso-rob)	254, 352	623/625	317/-	30
13.98	Isorhamnetin-3-O-rutinoside [a] (Iso-rut)	254, 352	623/625	317/-	30
14.12	Isorhamnetin-3-O-galactoside [b] (Iso-gal)	254, 352	477/479	317/-	30
14.38	Isorhamnetin-3-O-glucoside [b] (Iso-glu)	254, 352	477/479	317/-	30
15.05	Isorhamnetin 3-O-malonygalactoside [b] (Iso-malgal)	254, 352	563/565	317/-	30
15.36	Isorhamnetin 3-O-6"-malonygalactoside [b] (Iso-6"-malgal)	254, 352	563/565	317/-	30

The abbreviation used for each compound is shown in brackets.

[a] Identification on the basis of the UV-vis spectrum and specific mass fragments of the standard compounds.

[b] Tentative identification on the basis of the UV-vis spectrum, MS, and HPLC/UHPLC retention times and published data.

A mix of standard compounds with one standard of each family (arbutin (Arb), 5-caffeoylquinic acid (5-Caf-qui), catechin (Cat) and quercetin-3-O-glucoside (Q-glu)) was injected once every ten injections to control stability during the injection set. The areas of all peaks selected in the chromatograms of samples were normalized against the area of the corresponding standard of the same family of compounds injected in the sample set. The ratios of the peaks were then put into a table and used to build the PCA models. The weight of variables was normalized by the ratio of their standard deviation in data set. Scores and loadings in the bi-plots obtained in the PCA model were used to determine whether the phenolic compound profiles of each juice sample was sufficient to distinguish between the juice of pear varieties and between the puree of peach varieties, as well as to identify markers for each variety.

In the model built from the chromatograms of peach purees, the first two PCs (PC1 and PC2) were selected to provide the highest variation of data objects (42% PC1 and 32 % PC2). The scores of the PCA bi-plot (Figure 3) produced a moderate separation through PC1 of the commercial (C) and freshly blended (FB) purees of both the Miraflores (M) and Spring Lady (SL) cultivars. The loadings of the variables in the plot show that the separation was based on flavonol glycosides (Q-gal, Q-gluc, Q-rut and K-rut) against the other phenolic compounds (Pearson correlation between -0.68, -0.12, $p<0.05$). The separation between the cultivars was better in commercial (C) purees than in freshly blended (FB). The sample of freshly blended Miraflores puree from batch four (FB-M-4) was crossed in PCA model with samples of Commercial Spring Lady puree (C-SP). The FB-M-4 sample already showed lower separation in the PCA model by NMR. This observation could be attributed to the fact that sampling is never as thorough in a fruit batch as in a juice batch because the latter includes a homogenization step. In contrast to the former model with primary metabolites, this new model produced separation between commercial (C) and the freshly blended (FB) peach purees. Separation between these two types of commercial (C) and freshly blended (FB) purees was performed along PC2. Freshly blended samples (FB) samples showed a greater concentration of phenolic compounds than commercial samples (C). These results are in accordance with the degradation of phenolic compounds during the thermal and blending steps of fruit processing.[20, 23, 30].

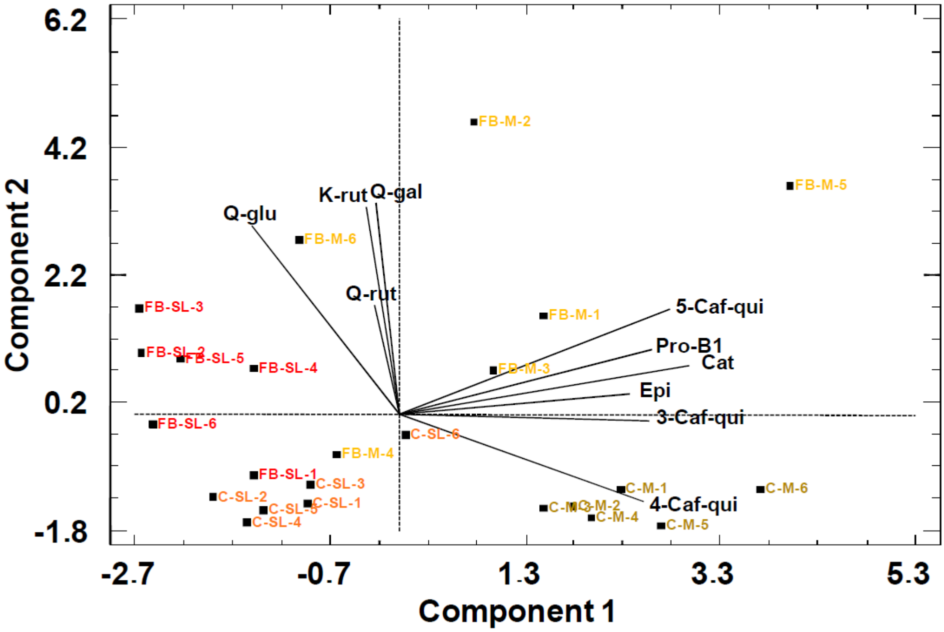


Figure 3. PCA bi-plot of peach purees with phenolic compounds as statistical variables. FB: Freshly blended, C: Commercial, SL: Spring Lady cultivar, and M: Miraflores cultivar. Ending number is the batch number. PC1: 42%, PC2: 32%.

The scores of the PCA bi-plot for pear juices (Figure 4) showed an improved separation over the model of primary metabolites obtained by ^{1}H NMR. In the model built from the phenolic compounds constituents of pear juices, isorhamnetin 3-O-malonygalactoside and Isorhamnetin 3-O-6''-malonygalactoside [b] (Iso-6''-malgal) were not included because they are not present in the juice of the C variety. In the model plotted, the first two PCs explained 96% of the information contained in the data (71% PC1 and 25% PC2). The hydroxycinnamic acids (3-Caf-qui, 5-Caf-qui, 4-Caf-qui) were strongly correlated among them and with arbutin (Arb), also in PC1 they were correlated with isorhamnetin glycosides (Iso-rut, Iso-gal, Iso-glu). In contrast, quercetin galactoside and glucoside (Q-gal and Q-glu) were anti-correlated with hydroxycinnamic acids (3-Caf-qui, 5-Caf-qui, 4-Caf-qui) through PC1, which allowed the separation of Alejandrina (AJ) from the other two cultivars. The negative correlation between isorharmnetin glycosides (Iso-rut, Iso-gal) and quercetin glycosides (Q-gal and Q-glu) in the PC2 allowed the separation between the Blanquilla (BJ) and Conference (CJ) cultivars.

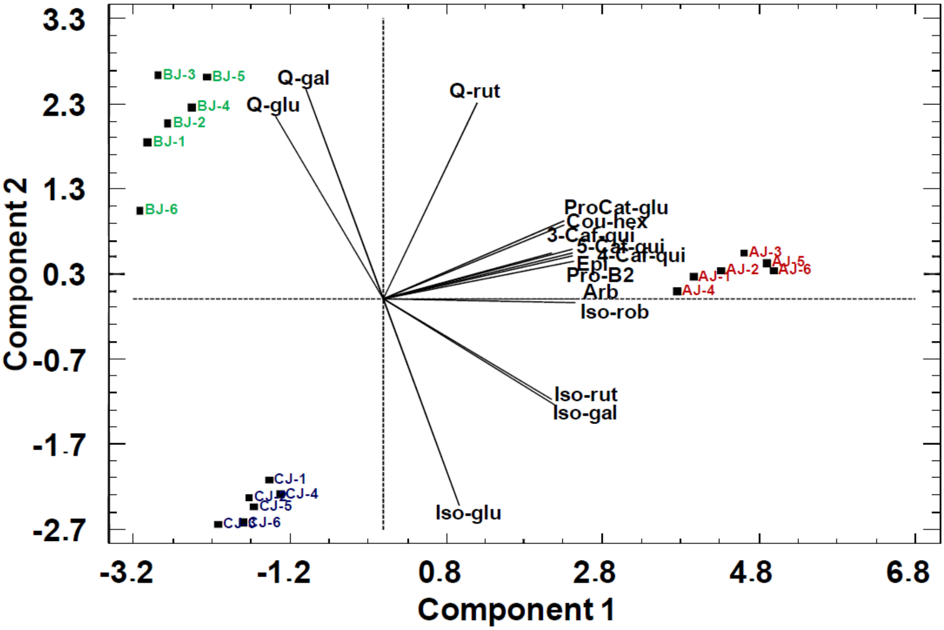


Figure 4. PCA bi-plot of pear juices with phenolic compounds as statistical variables. AJ: Alejandrina juice, CJ: Conference juice, and BJ: Blanquilla juice. PC1: 71%, PC2: 25%.

3.3. Combination of ^{1}H NMR and UPLC-PDA-FLR-MS/MS chemometric analysis.

The PCA models of peach puree and pear juices (Figure 5 and 6) were built by combining data of primary metabolites and phenolic compounds, in order to evaluate the improvement in separation obtained. In both models, the first two PCs provided the highest variation of data objects (PC1 and PC2, respectively, accounting for 37% and 22% of the information for peach purees, and 58% and 32% for pear juices).

The scores of the PCA bi-plot combination for peach purees (Figure 5) substantially improved the separation with respect to the separate models for primary metabolites and phenolic compounds. The separation was achieved through PC1 component between the Miraflores (M) and Spring Lady (SL) cultivars both commercial (C) and freshly blended (FB) purees. Only the outlier FB-M-4 continued to be separated from the other samples of freshly blended puree from Miraflores cultivar.

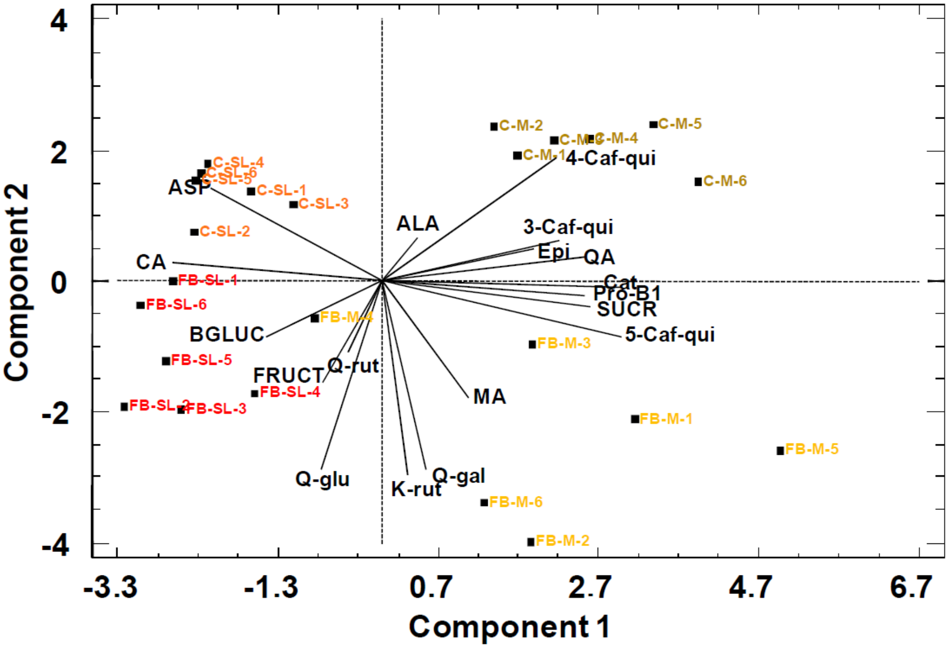


Figure 5. PCA bi-plot of peach purees with primary metabolites and phenolic compounds as statistical variables. FB: Freshly blended, C: Commercial, SL: Spring Lady cultivar, and M: Miraflores cultivar. Ending number is the batch number. PC1: 37%, PC2: 22%.

The loadings of the variables in this plot showed that the separation was based on asparagine (ASP) and citric acid (CA) against sucrose (SUCR), quinic acid(QA), epicatechin (Epi), catechin (Cat) and various hydroxycinnamics acids (Pearson correlation between -0.68 and -0.12, $p<0.05$). The inclusion of the phenolic compounds in the primary metabolite model allowed the separation of commercial (C) and freshly blended (FB) purees.

Regarding the pear juices (Figure 6), the PCA bi-plot combination improved the density of scores of juice from the Blanquilla cultivar, but did not affect the separation obtained in the previous models. Indeed, the three cultivars were separated along the PC1.

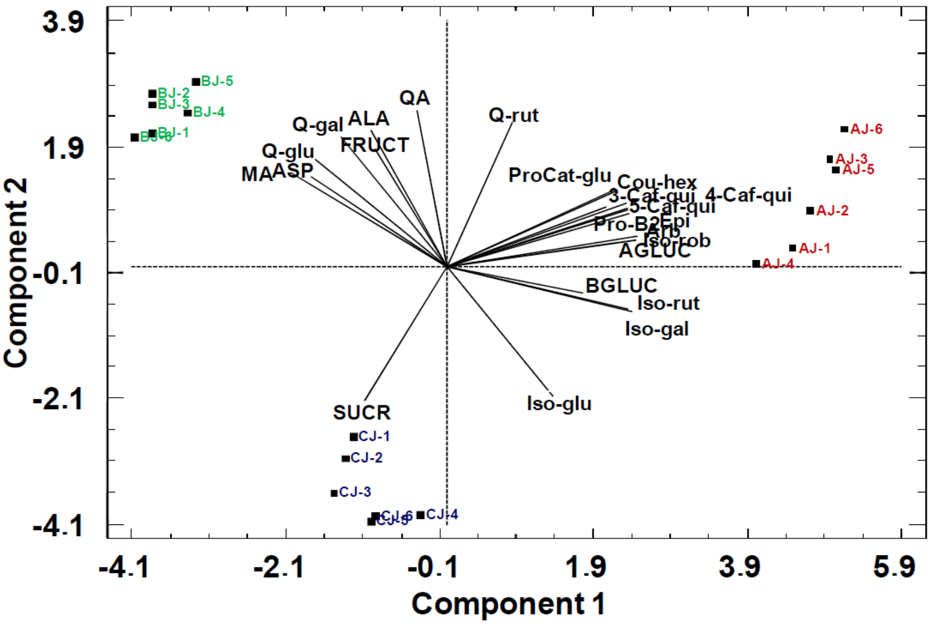


Figure 6. PCA bi-plot of pear juices with primary metabolites and phenolic compounds as statistical variables. AJ: Alejandrina juice, CJ: Conference juice, and BJ: Blanquilla juice. PC1: 58%, PC2: 32%.

4. Conclusions.

Our present study shows the approach of authenticate single-varietal peach purees and pear juices on the basis of primary metabolites and phenolic compounds. The PCA of primary metabolites, from ^{1}H-NMR data, allowed the discrimination between the peach varieties and pear varieties, but not allowed discriminate between processed and non-processed purees. On the other hand, PCA approaches from phenolic compounds analyzed by UPLC-PDA-MS/MS discriminated between varieties in peach purees and pear juices and also permitted discriminate between processed and non processed peach purees. The PCA combined data of primary metabolites and phenolic compounds improved the separation of clusters in all cases.

Abbreviations used.

^{1}H-NMR, hydrogen nuclear magnetic resonance; UPLC-PDA-MS/MS, ultrahigh-performance liquid chromatography coupled with a photo diode array and a tandem mass spectrometer detectors; PCA, principal component analysis; LC-PDA-MS, liquid chromatography coupled with a photo diode array and a mass spectrometer detectors; MeOH, Methanol; ACN, acetonitrile; HAc, acetic acid; HFor, formic acid; FB, Freshly blended; C, Commercial; SL, Spring Lady cultivar; M, Miraflores cultivar; AJ, Alejandrina juice; CJ, Conference juice; BJ, Blanquilla juice; IS, internal standard; ALA, alanine; QA, quinic acid; CA, citric acid; ASP, asparagine; βGLC, β-glucose; FRU, β-D-fructopyranose; MA, malic acid; αGLC, α-glucose; SUCR, sucrose; Arb, Hydroquinone β-D-glucopyranoside or Arbutin; ProCat-glu, Protocatechuic aldehyde monoglucuronide; 3-Caf-qui, 3-Caffeoylquinic acid; Cou-hex, Coumaroyl-hexose; Pro-B1, Procyanidin-B1; 5-Caf-qui, 5-Caffeoylquinic acid; Cat, (+)-Catechin; 4-Caf-qui, 4-Caffeoylquinic acid; Pro-B2, Procyanidin-B2; 5-p-Cou-qui, 5-p-Coumaroylquinic acid; Epi, (-)-Epicatechin; Q-rut, Quercetin-3-O-rutinoside; Q-gal, Quercetin-3-O-galactoside; Q-glu, Quercetin-3-O-glucoside; K-rut, Kaempferol 3-O-rutinoside; Iso-rob, Isorhamnetin-3-O-robinioside; Iso-rut, Isorhamnetin-3-O-rutinoside; Iso-gal, Isorhamnetin-3-O-galactoside; Iso-glu, Isorhamnetin-3-O-glucoside; Iso-malgal, Isorhamnetin 3-O-malonygalactoside; Iso-6''-malgal, Isorhamnetin 3-O-6''-malonygalactoside.

References.

[1] AIJN, European Fruit Juice Association, (2012).

[2] K. Robards, M. Antolovich, Analyst, 120 (1995) 1.

[3] M. Cuny, E. Vigneau, G. Le Gall, I. Colquhoun, M. Lees, D. Rutledge, Analytical and Bioanalytical Chemistry, 390 (2008) 419.

[4] M.R. Johnston, F. Kauffman, Journal of Food Quality, 8 (1985) 81.

[5] L. Vaclavik, A. Schreiber, O. Lacina, T. Cajka, J. Hajslova, Metabolomics, 8 (2012) 793.

[6] L. Mondello, P.Q. Tranchida, P. Dugo, G. Dugo, Mass Spectrometry Reviews, 27 (2008) 101.

[7] G. Downey, Journal of Near Infrared Spectroscopy, 4 (1997) 47.

[8] N. Ogrinc, I.J. Košir, J.E. Spangenberg, J. Kidrič, Analytical and Bioanalytical Chemistry, 376 (2003) 424.

[9] P.S. Belton, I.J. Colquhoun, E.K. Kemsley, I. Delgadillo, P. Roma, M.J. Dennis, M. Sharman, E. Holmes, J.K. Nicholson, M. Spraul, Food Chemistry, 61 (1998) 207.

[10] M. Spraul, B. Schütz, P. Rinke, S. Koswig, E. Humpfer, H. Schäfer, M. Mörtter, F. Fang, U.C. Marx, A. Minoja, Nutrients, 1 (2009) 148.

[11] F. Zeng, J. Ou, Y. Huang, Q. Li, G. Xu, Z. Liu, S. Yang, Food Analytical Methods, 8 (2015) 428.

[12] R. Zadernowski, S. Czaplicki, M. Naczk, Food Chemistry, 112 (2009) 685.

[13] M.I. Gil, F.A. Tomás-Barberán, B. Hess-Pierce, A.A. Kader, Journal of Agricultural and Food Chemistry, 50 (2002) 4976.

[14] B.A. Cevallos-Casals, D. Byrne, W.R. Okie, L. Cisneros-Zevallos, Food chemistry, 96 (2006) 273.

[15] C. Andreotti, D. Ravaglia, A. Ragaini, G. Costa, Annals of Applied Biology, 153 (2008) 11.

[16] D. Capitani, A.P. Sobolev, A. Tomassini, F. Sciubba, F.R. De Salvador, L. Mannina, M. Delfini, Journal of Agricultural and Food Chemistry, 61 (2012) 1718.

[17] D. Tanrıöven, A. Ekşi, Food Chemistry, 93 (2005) 89.

[18] L.-Z. Lin, J.M. Harnly, Journal of Agricultural and Food Chemistry, 56 (2008) 9094.

[19] C. Kevers, J.l. Pincemail, J. Tabart, J.-O. Defraigne, J. Dommes, Journal of Agricultural and Food Chemistry, 59 (2011) 6165.

[20] G.A. Spanos, R.E. Wrolstad, Journal of Agricultural and Food Chemistry, 40 (1992) 1478.

[21] Y.-J. Hong, D.M. Barrett, A.E. Mitchell, Journal of Agricultural and Food Chemistry, 52 (2004) 2366.

[22] D.K. Asami, Y.J. Hong, D.M. Barrett, A.E. Mitchell, Journal of the Science of Food and Agriculture, 83 (2003) 56.

[23] S. Basak, H. Ramaswamy, Journal of Texture Studies, 29 (1998) 587.

[24] Y.H. Hui, Blackwell Publishing, (2006).

[25] M.N. Clifford, K.L. Johnston, S. Knight, N. Kuhnert, Journal of Agricultural and Food Chemistry, 51 (2003) 2900.

[26] B. Abad-Garcia, S. Garmon-Lobato, L.A. Berrueta, B. Gallo, F. Vicente, Journal of Mass Spectrometry : JMS, 44 (2009) 1017.

[27] U. Vrhovsek, D. Masuero, M. Gasperotti, P. Franceschi, L. Caputi, R. Viola, F. Mattivi, Journal of agricultural and food chemistry, 60 (2012) 8831.

[28] M. Scordino, L. Sabatino, A. Muratore, A. Belligno, G. Gagliano, Journal of Food Quality, 35 (2012) 255.

[29] D. Ferreira, S. Guyot, N. Marnet, I. Delgadillo, C.M. Renard, M.A. Coimbra, Journal of agricultural and food chemistry, 50 (2002) 4537.

[30] J. Lee, R.W. Durst, R.E. Wrolstad, Journal of Food Science, 67 (2002) 1660.

APPENDIX A. supplementary data.

Phenolic compounds chromatograms.

Table S-1 UV max of the phenolic compounds identified in the samples.

Peak number	Compound	UV max (nm)
1	Hydroquinone β-D-glucopyranoside or Arbutin [a] (Arb)	283
2	Protocatechuic aldehyde monoglucuronide [b] (ProCat-glu)	268
3	3-Caffeoylquinic acid [b] (3-Caf-qui)	240, 326
4	Coumaroyl-hexose [b] (Cou-hex)	316
5	Procyanidin-B1 [a] (Pro-B1)	278
6	5-Caffeoylquinic acid [a] (5-Caf-qui)	240, 326
7	(+)-Catechin [a] (Cat)	278
8	4-Caffeoylquinic acid [b] (4-Caf-qui)	240, 326
9	Procyanidin-B2 [a] (Pro-B2)	278
10	5-*p*-Coumaroylquinic acid [b] (5-p-Cou-qui)	235,312
11	(-)-Epicatechin [a] (Epi)	278
12	Quercetin-3-O-rutinoside [a] (Q-rut)	255, 353
13	Quercetin-3-O-galactoside [a] (Q-gal)	255, 353
14	Quercetin-3-O-glucoside [a] (Q-glu)	255, 353
15	Kaempferol 3-O-rutinoside [a] (K-rut)	265, 347
16	Isorhamnetin-3-O-robinioside [b] (Iso-rob)	254, 352
17	Isorhamnetin-3-O-rutinoside [a] (Iso-rut)	254, 352
18	Isorhamnetin-3-O-galactoside [b] (Iso-gal)	254, 352
19	Isorhamnetin-3-O-glucoside [b] (Iso-glu)	254, 352
20	Isorhamnetin 3-O-malonygalactoside [b] (Iso-malgal)	254, 352
21	Isorhamnetin 3-O-6"-malonygalactoside [b] (Iso-6"-malgal)	254, 352

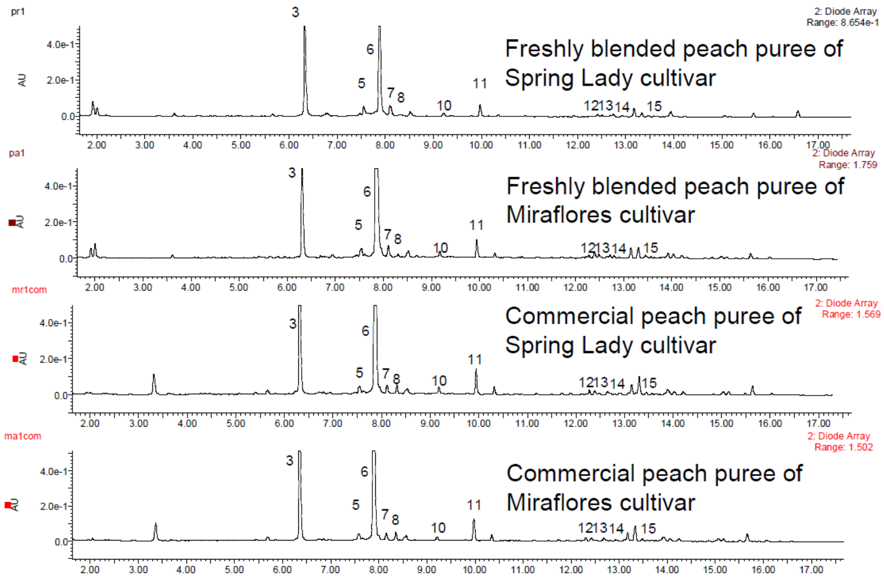


Figure S-1. Chromatograms at 350 + 280 +320 nm peach purees. Numbered according the table S-1.

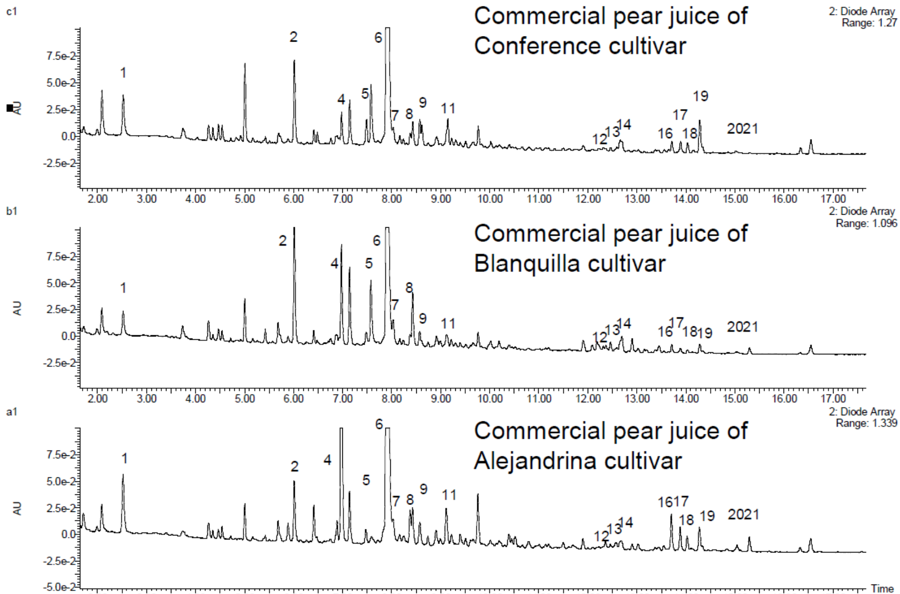


Figure S-2. Chromatograms at 350 + 280 +320 nm commercial pear juices. Numbered according the table S-1.

^{1}H NMR Spectra.

Table S-2. Assignment and identification of the ^{1}H-NMR selected signals and their shifts.

Signal number	Compound	Assignment	^{1}H (ppm)
1	Alanine (ALA)	β-CH_3	1.49
2	Quinic acid (QA)	CH_2-1,1'; CH_2-5,5'	1.7
3	Citric acid (CA)	α,γ-CH	2.3
4	Asparagine (ASP)	β, β'-CH_2	2.7
5	β-Glucose (βGLC)	CH-2	2.95
6	β-D-Fructopyranose(FRU)	CH_2-6,6'	3.85
7	Malic acid (MA)	β, β'-CH_2	4.1
8	α-Glucose (αGLC)	CH-1	4.95
9	Sucrose (SUCR)	GLC CH-1	5.15
10	Maleic acid (IS)	2CH	5.9

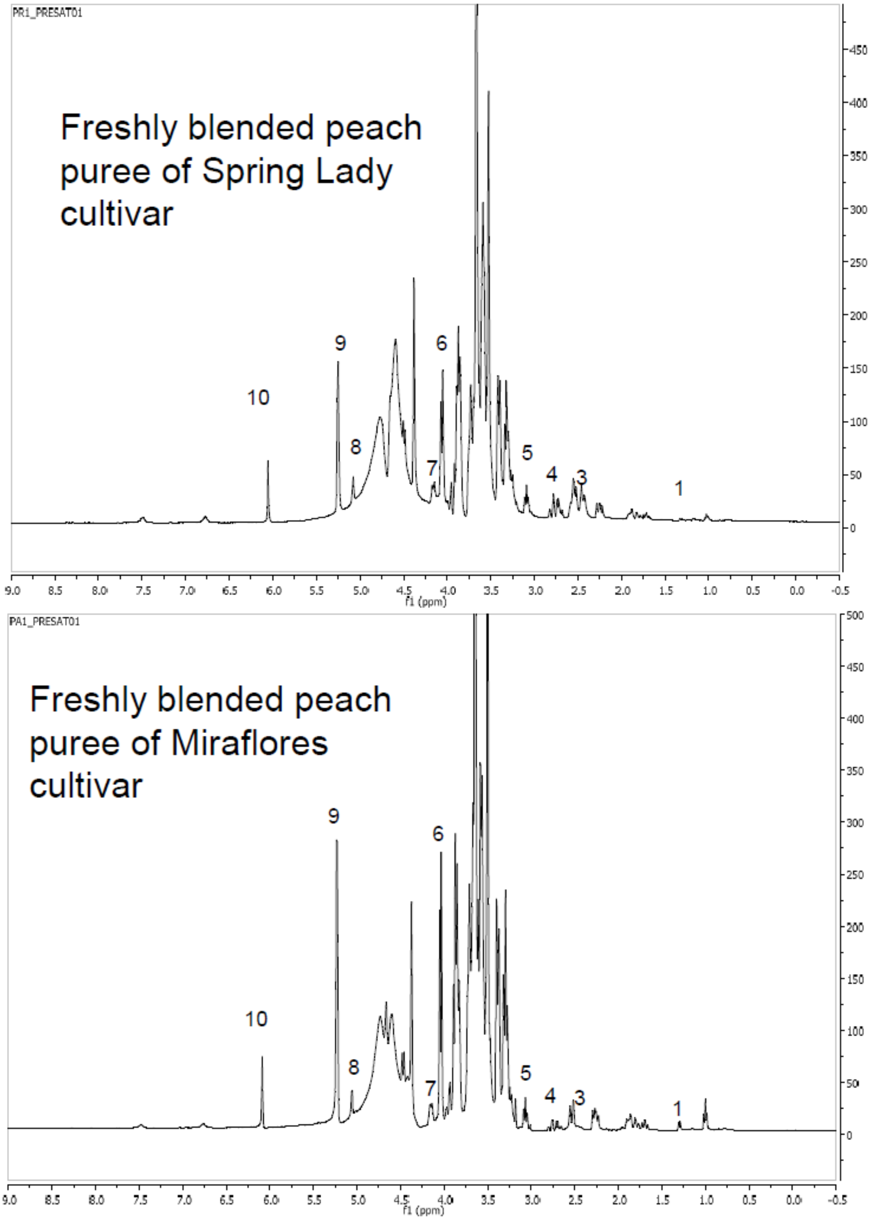


Figure S-3. ^{1}H NMR spectra of freshly blended peach purees. Numbered according the table S-2.

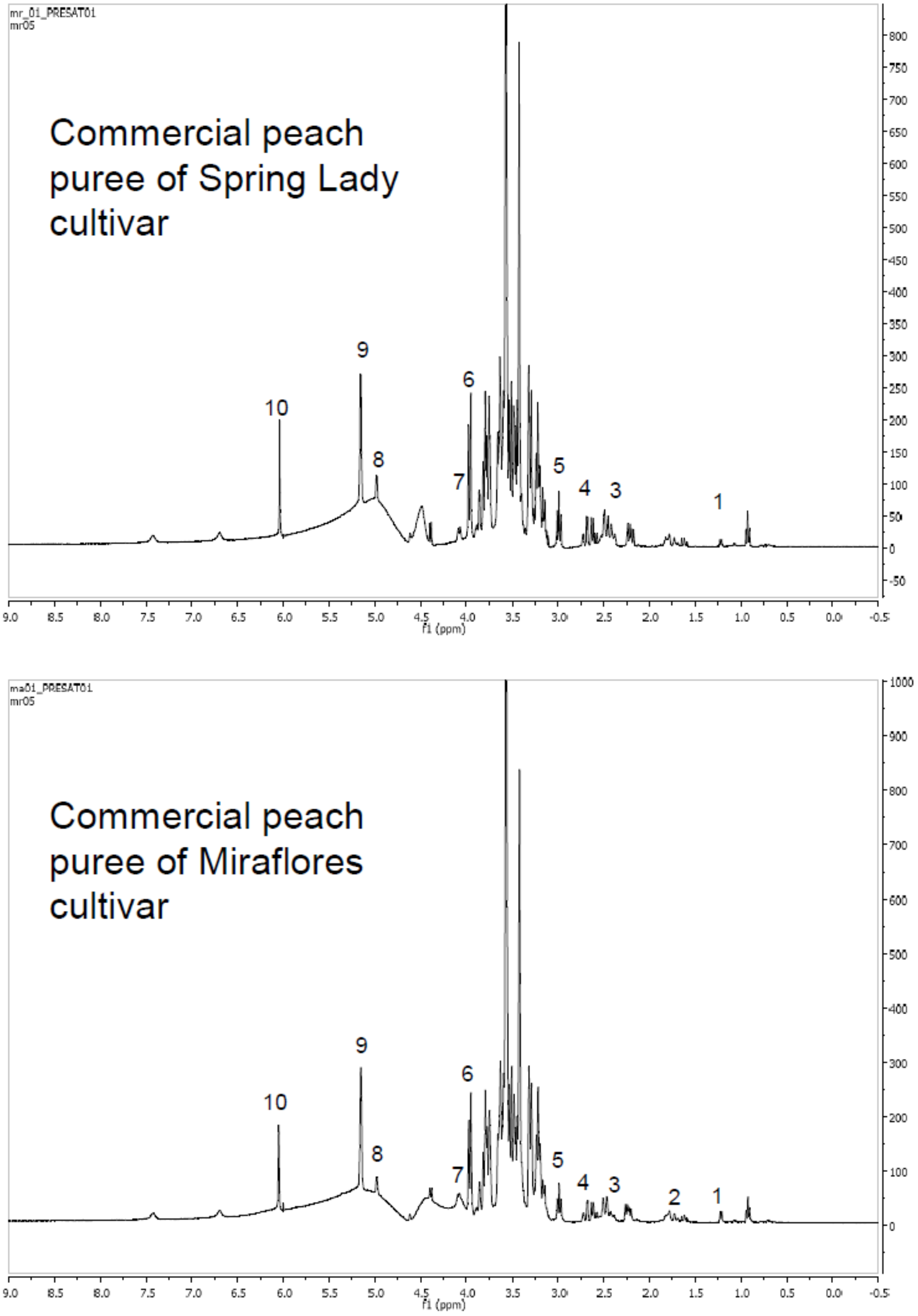


Figure S-4. ^1H NMR spectra of commercial peach purees. Numbered according the table S-2.

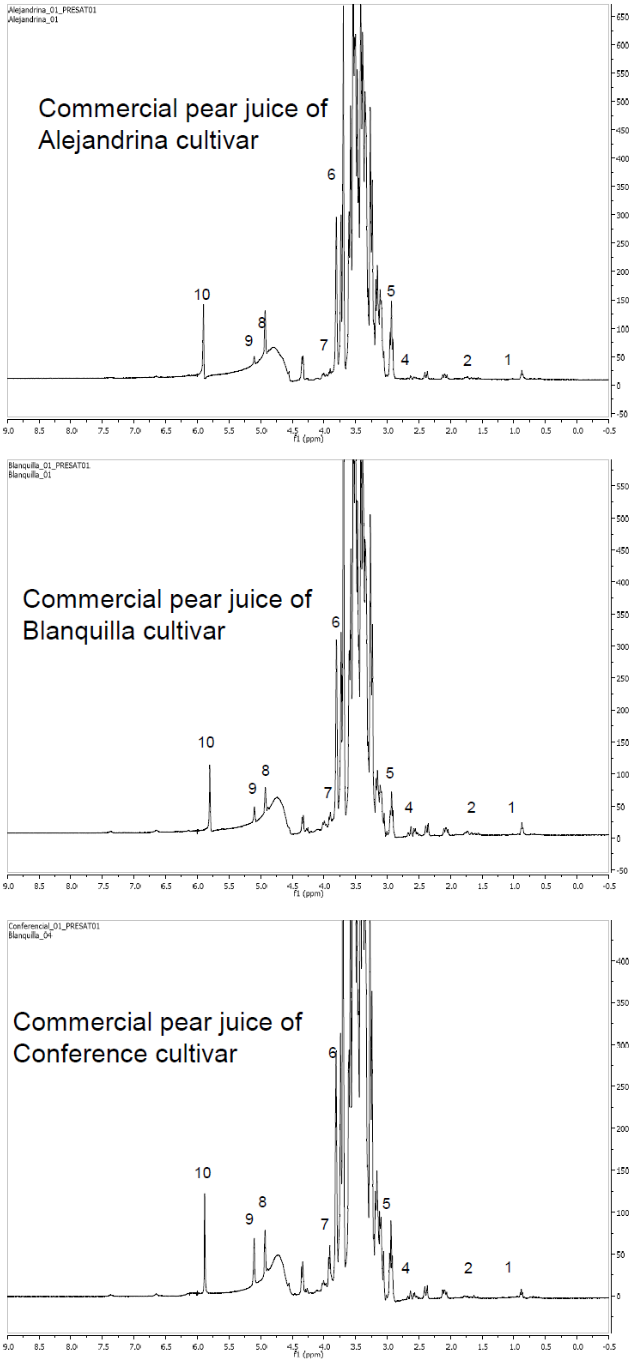


Figure S-5. ^{1}H NMR spectra of commercial pear juices. Numbered according the table S-2.

Statistical analysis.

Table S-3. Oneway Anova p-value cut-off:.0.05, on peach puree samples

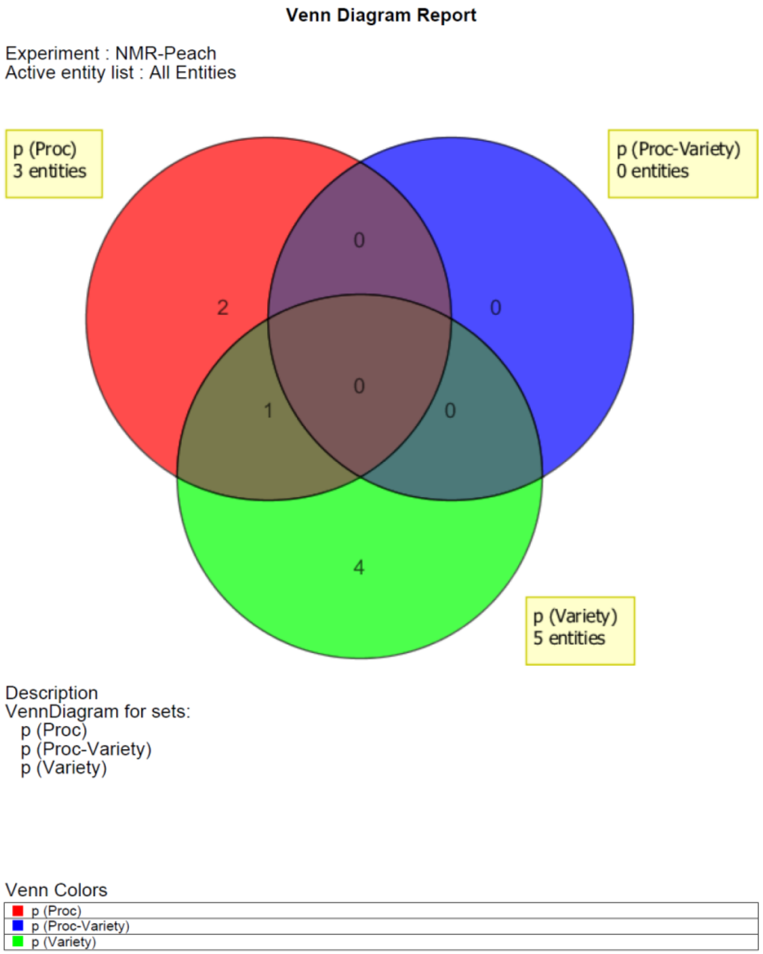
Compound	p	FC ([PM] vs [PSL])	FC ([PM] vs [UnPM])	FC ([PM] vs [UnPSL])	FC ([PSL] vs [UnPM])	FC ([PSL] vs [UnPSL])	FC ([UnPM] vs [UnPSL])
SUCR	1.42E-04	**1.48**	-1.02	**1.43**	**-1.51**	-1.03	**1.46**
FRU	4.94E-02	-1.12	-1.24	-1.22	-1.11	-1.08	1.02
bGLUC	2.06E-02	-1.08	-1.05	**-1.25**	1.03	-1.15	-1.19
ASP	3.42E-05	**-1.42**	**1.45**	-1.22	**2.05**	1.16	**-1.77**
CA	1.06E-06	**-1.95**	1.14	**-2.27**	**2.22**	-1.17	**-2.59**
MA	4.35E-03	1.15	-1.23	-1.11	**-1.42**	**-1.28**	1.11
QA	1.46E-02	1.33	1.14	**1.45**	-1.17	1.09	1.27
3-Caf-qui	1.15E-02	**1.46**	1.23	1.29	-1.18	-1.13	1.05
5-Caf-qui	1.27E-08	**1.89**	-1.17	**2.06**	**-2.21**	1.09	**2.41**
4-Caf-qui	1.10E-08	**2.99**	**2.74**	**5.85**	-1.09	**1.96**	**2.13**
Pro-B1	1.71E-09	**4.43**	-1.08	**10.13**	**-4.79**	**2.28**	**10.94**
Cat	2.03E-07	**2.47**	-1.04	**4.55**	**-2.56**	**1.84**	**4.72**
Epi	1.29E-03	1.49	1.10	**3.21**	-1.36	**2.15**	**2.93**
Q-gal	7.63E-06	-1.39	**-4.44**	**-2.61**	**-3.19**	**-1.87**	1.70
Q-glu	9.43E-07	**-2.04**	**-3.80**	**-4.60**	**-1.87**	**-2.26**	-1.21
K-rut	7.12E-06	1.07	**-2.66**	**-2.45**	**-2.86**	**-2.63**	1.09

In bold significant compounds ($p<0.05$) in Tuckey HSD *post-hoc*

Table S-4. One way Anova p-value cut-off:.0.05, on pear juice samples.

Compound	p	FC ([Alej] vs [Blanq])	FC ([Alej] vs [Conf])	FC ([Blanq] vs [Conf])
SUCR	1.68E-10	**-1.83**	**-4.44**	**-2.43**
aGLUC	4.09E-05	**1.75**	**1.49**	-1.17
bGLUC	4.54E-03	**1.62**	1.22	-1.33
FRUCT	7.30E-03	-1.24	1.10	**1.37**
ASP	4.36E-08	**-3.38**	-1.30	**2.61**
MA	4.33E-04	**-1.55**	-1.07	**1.44**
QA	3.93E-08	**-1.33**	**2.16**	**2.89**
ALA	5.18E-06	**-1.67**	**1.55**	**2.58**
Arb	3.25E-10	**2.28**	**1.86**	**-1.22**
ProCat-glu	1.64E-15	**2.70**	**5.22**	**1.93**
3-Caf-qui	4.02E-13	**3.01**	**3.81**	**1.26**
Cou-hex	6.40E-20	**3.56**	**11.44**	**3.21**
5-Caf-qui	2.88E-04	**1.39**	**1.44**	1.03
4-Caf-qui	1.32E-13	**2.92**	**3.30**	1.13
Pro-B2	3.21E-10	**2.44**	**2.52**	1.03
Epi	8.23E-10	**2.40**	**2.63**	1.10
Q-rut	3.35E-10	1.22	**9.08**	**7.46**
Q-gal	9.36E-11	**-1.97**	**1.39**	**2.74**
Q-glu	1.12E-07	**-1.76**	-1.00	**1.75**
Iso-rob	4.26E-12	**5.24**	**3.23**	**-1.62**
Iso-rut	4.66E-11	**6.33**	**1.60**	**-3.96**
Iso-gal	7.60E-15	**5.81**	**1.52**	**-3.83**
Iso-glu	3.14E-13	**3.73**	**-1.27**	**-4.75**

In bold significant compounds ($p<0.05$) in Tuckey HSD *post-hoc* analysis



Venn Diagram Report

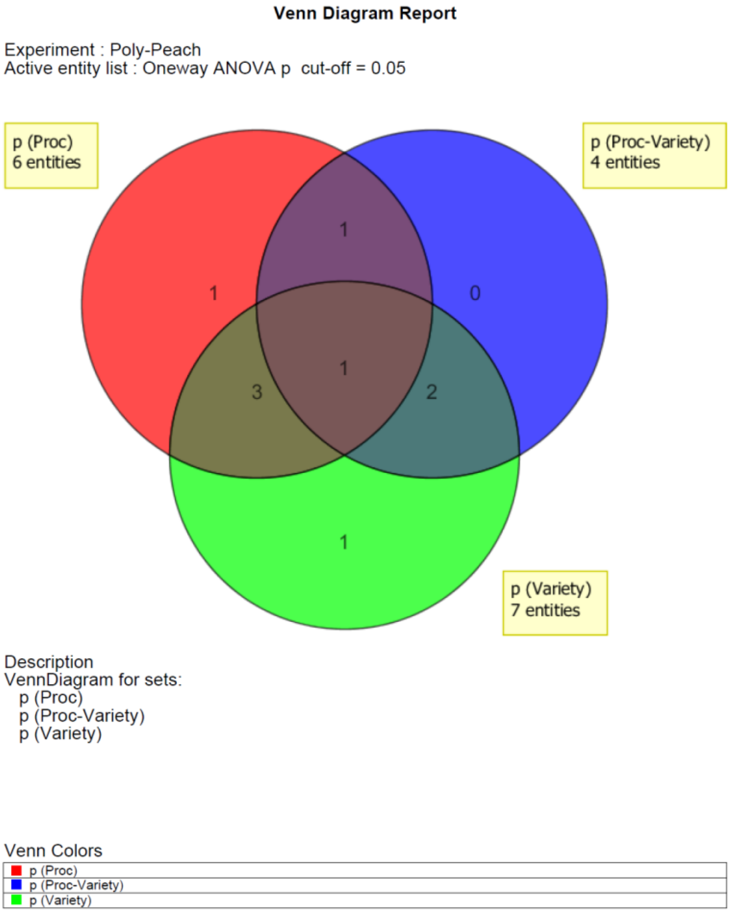
Experiment : NMR-Peach
Active entity list : All Entities

Description
VennDiagram for sets:
p (Proc)
p (Proc-Variety)
p (Variety)

Venn Colors

🟥 p (Proc)
🟦 p (Proc-Variety)
🟩 p (Variety)

Figure S-6. Venn diagram report of primary metabolites peach



Venn Diagram Report

Experiment : Poly-Peach
Active entity list : Oneway ANOVA p cut-off = 0.05

Description
VennDiagram for sets:
p (Proc)
p (Proc-Variety)
p (Variety)

Venn Colors

🟥	p (Proc)
🟦	p (Proc-Variety)
🟩	p (Variety)

Figure S-7. Venn diagram report of phenolic compounds in peach

CHAPTER 4. GENERAL DISCUSSION

An UPLC–PDA–MS/MS analytical method that allows the simultaneous determination of epoxycarotenoids, hydroxycarotenoids and carotenes in monovarietal freshly made and industrially processed fruit juices was developed. Up to 27 carotenoids were tentatively identified in apple, peach and pear products, based on chromatographic retention time, UV-vis absorption spectra and MS/MS. This study is the first to describe the separation of epoxycarotenoids by UPLC and greatly improves the previously reported HPLC methods to analyse the carotenoids present in fruit or fruit products [1,2]. Simultaneously various solvents and mixtures of solvents were tested, showing methanol extraction the maximum level of extraction of carotenoids.

The method developed was applied to freshly squeezed juices of several apple varieties to assess its capacity to distinguish between monovarietal juices, and to study the changes in the carotenoid profile in commercial and freshly made monovarietal fruit products (peach purees and pear juices). The complex carotenoid profile of freshly squeezed apple juices was similar than described previously for fresh fruit [3], and the method allowed characterise and differentiate 6 varieties of apple cultivars.

The carotenoids of freshly blended peach purees and commercial peach purees were identified and quantified [4]. We found that the 5,6-epoxycarotenoids are the main carotenoids defining the profile in the freshly made samples, while they did not appear in the commercial samples. Comparing the content of violaxanthin isomers, it was higher in the freshly blended puree, whereas the content of auroxanthin epimers (derived from violaxanthin isomers) was higher in the commercial juices. Neoxanthin isomers and antheraxanthin rearrangements exhibited the same behaviour, promoting the presence of neochrome epimers and the mutatoxanthin epimers, respectively. These differences can be attributed to the processing time and the temperature used for pasteurisation that increase isomerisation of 5,6- to 5,8-epoxides in the presence of acid media [5, 6]. Comparing freshly blended purees and commercial purees, the relative ratios of the other carotenoids such as hydroxycarotenoids and carotenes, remained unaltered. It is important to note that ζ-carotene was tentatively identified and quantified, becoming the first time that this carotene has been reported in peach samples.

An UPLC–PDA–MS/MS analytical method that allows the simultaneous determination of several chlorophyll and derivatives was developed. Up to 48 chlorophylls and derivatives were identified, based on chromatographic retention time, UV-vis absorption spectra and MS/MS combining APCI and ESI ionisations. The method allowed the proper separation of the chlorophyll derivatives obtained from demetalitation, dephytilation, decarbomethoxylation, epimerisation and copperisation in less than 12 min. This study is the first to describe the separation of all these chlorophyll derivatives by UPLC and greatly improves the previously reported HPLC methods [7-10]. This improvement is due to greater peak efficiencies obtained by UPLC and the use of ionic pairing and HSS T3 column to resolve the zone of dephytylated and chlorin compounds.

The method developed was applied to assess the fate of chlorophylls in processed vegetable products. First, the method was used to analyse various tea types to assess the capacity of the analytical method to distinguish between them and their level of processing. Several publications have described the profile of chlorophyll derivatives in tea [11, 12, 13], but an accurate description of the amount of chlorophyll and their derivatives to be used as quality indicator has not been achieved so far. Here we observed the different grade of processing between commercial common teas against luxury teas, since the content of chlorophylls and their derivatives was higher in the teas from specialty shops. In addition, an approach to differentiate among varieties of tea such as, Matcha, Sencha, Silver Needle, Kenya and Pur-Eh showed encouraging results.

Secondly, the developed methodology has also been applied in other samples obtained from green vegetables, such as purees, jams, juices and other vegetable products. Contrary to happened in the tea samples in any of the samples were detected chlorophylls or chlorophyllides. This can be in accordance with publications describing an almost complete conversion of chlorophyll to pheophytinized derivatives during prolonged heat treatments in acidic conditions[15]. In these samples were determined mainly pehophytin, pheophorbides and pyro derivatives thereof. Although not stated in the ingredients, several of the products analysed in the study presented copper pheophytins typical from the coloring E-141 (i), such as Cu-pheophytins, Cu-pyropheophytins, Cu-pheophorbides and Cu-pyropheophorbide [16].

In the second chapter, the thesis focuses on the characterisation of in processed fibres from the juice industry. The nine selected fibres were apple, peach, pear, orange peel and flesh, tangerine peel and flesh, lemon flesh and carrot. In a first approach, the study focuses on the phenolic compounds. The content of phenolic compounds was determined in nine industrially processed fibres derived from the juice industry. In addition, to allow the comparison of the profiles and contents in the fresh fruit and fibres, extractable and bound phenolic compounds were analysed in lyophilized peel and pulp from fresh fruit.

A different behaviour was observed when comparing industrial fibres with lyophilized peel and flesh, depending on the family of compounds assessed. The phenolic profiles in fibres were found to be consistent with those in the lyophilised material from fresh fruits. In contrast, the content of flavan-3-ols in fibres was lower than in lyophilized peel and flesh in non-citrus fruits. These results could be attributed to the degradation of flavan-3-ols during processing [17, 18]. In citrus fruits, lemon showed significantly lower concentrations of phenolic compounds in fibres than in peel and lyophilised flesh. These findings were consistent with the significantly greater content of 5-hydroxymethylfurfural (5-HMF) in lemon fibres, which can be explained by degradation caused by the temperature reached during processing [19]. In addition, we examined bound phenolic compounds remaining in solid residues. Bound phenolic acids and bound proanthocyanidins differed in behaviour depending on the origin of the fibre or the lyophilised material. In the citrus and carrot fibres, the concentration of condensed tannins was considerably lower than in the other fibres, confirming the results previously reported in bibliography [20].

In a second approach, in the study were analysed the content of primary and secondary metabolites in nine types of industrially processed fibres derived from the juice industry. Regarding primary metabolites, the sugar content ranged from 21.6 mg/g in lemon to 290 mg/g in orange peel and lower mass organic acid content ranged from 25.0 mg/g in pear to 250 mg/g in lemon. The content and profile of fatty acids were constant during fibre processing, ranging from 0.5 to 1.46%. In general, the industrial process did not greatly affect the original profile of primary metabolites present. The processing of fruit to prepare juices and purees does not significantly affect some secondary metabolites or minor compounds such as sterols or alkanes in the resulting fibres [21, 22]. Total plant sterols ranged from 0.51 to 1.66 μg/g in the nine types of fibre studied. The highest content was in orange fibre (1.66 μg/g) and the lowest in pear fibre (0.51 μg/g). β-Sitosterol and stigmasterol were found in all fibres, being the major phytosterol (0.41 to 1.01 μg/g) in all cases. Triterpenes were found only in non-citrus fibre and in carrot fibre. The predominant triterpene was ursolic acid, followed by oleanolic acid and α-amyrin, and the maximum contents were found in apple fibre. The profiles and contents of these three compounds are similar to those reported previously in fresh fruit [23, 24] and consistent with the findings of Geana et al., who described apple pomace as a suitable source of bioactive compounds such as ursolic and oleanolic acids [25].

In terms of total carotenoid content, carrot fibre showed the highest value in the single batch available. After carrot fibre, the fibres of orange flesh and tangerine peel showed the highest content of total carotenoid. The content and profile of carotenoids varied greatly in the six batches of orange flesh fibre. It should be noted that the profile of carotenoids depend greatly on the variety of the orange, the harvesting origin, and climatic factors [26]. The major carotenoids in orange and tangerine fruits are violaxanthin isomers, (all-*E*)-lutein, and (all-*E*)-β-cryptoxanthin [6, 27]. In the case of citrus fibre, we found high contents of (all-*E*)-β-cryptoxanthin and phytoene. Phytofluene, (all-*E*)-lutein, (all-*E*)-zeaxanthin, α-carotene, and two isomers of β-carotene (A and B) were present at lower concentrations. α-Carotene was particularly abundant in tangerine peel fibre, supporting the notion that this compound is present mainly in peel [28]. In addition, neochrome, auroxanthin and mutatoxanthin were also found in substantial quantities. We can explain the presence of auroxanthin and mutatoxanthin isomers, which are the epoxide–furanoid rearrangement products of violaxanthin and antheraxanthin, respectively [29]. In this case, the organic acids released during the industrial processing of fruit juices decrease the pH enough to promote rearrangements of 5,6-epoxide to 5,8-epoxide [5].

In the third chapter, we authenticated single-varietal peach purees and pear juices on the basis of primary metabolites and phenolic compounds analysis by ^{1}H-NMR and UPLC-PDA-MS/MS, respectively. NMR spectroscopy has been used to analyze primary metabolites (amino acids, organic acids, and sugars), while LC-PDA-MS has been successfully applied for the analysis of phenolic compounds in fruit samples [30-32]. After suitable pre-processing, the ^{1}H-NMR and chromatographic data were evaluated by PCA, both separately and pooled. The PCA of primary metabolites, from ^{1}H-NMR data, allowed the discrimination between the peach cultivar varieties (Miraflores and Spring Lady) and pear cultivar varieties (Alejandrina, Conference, and Blanquilla), but not allowed discriminate between processed and non-processed purees. On the other hand, PCA approaches from phenolic compounds analyzed by UPLC-PDA-MS/MS discriminated between varieties in peach purees and pear juices and also permitted discriminate between processed and non processed peach purees. The PCA combined data of primary metabolites and phenolic compounds improved the separation of clusters in all cases.

References.

[1] A.J. Meléndez-Martínez, I.M. Vicario, F.J. Heredia, Journal of Food Composition and Analysis, 22 (2009) 295.

[2] P. Molnár, M. Kawase, K. Satoh, Y. Sohara, T. Tanaka, S. Tani, H. Sakagami, H. Nakashima, N. Motohashi, N. Gyémánt, J. Molnár, Phytotherapy Research, 19 (2005) 700.

[3] C. Ampomah-Dwamena, S. Dejnoprat, D. Lewis, P. Sutherland, R.K. Volz, A.C. Allan, Journal of Experimental Botany, 63 (2012) 4497.

[4] M. Caller, G. Mackinney, Journal of Food Science., 30 (1965) 393.

[5] A. Asai, M. Terasaki, A. Nagao, Journal of Nutrition, 134 (2004) 2237.

[6] A.J. Meléndez-Martínez, I.M. Vicario, F.J. Heredia, Food Chemistry, 104 (2007) 169.

[7] Y. Suzuki, Y. Shioi, Journal of Agricultural and Food Chemistry, 51 (2003) 5307.

[8] L. Almela, J.A. Fernández-López, M.a.J. Roca, Journal of Chromatography A, 870 (2000) 483.

[9] J.-L. Lafeuille, S. Lefèvre, J. Lebuhotel, Journal of Agricultural and Food Chemistry, 62 (2014) 1926.

[10] C.H. Loh, B.S. Inbaraj, M.H. Liu, B.H. Chen, Journal of Agricultural and Food Chemistry, 60 (2012) 6108.

[12] R. Aparicio-Ruiz, K.M. Riedl, S.J. Schwartz, Journal of Agricultural and Food Chemistry, 59 (2011) 11100.

[13] A. Loranty, E. Rembiałkowska, E.A. Rosa, R.N. Bennett, Journal of Food Composition and Analysis, 23 (2010) 432.

[14] H. Horie, K. Kohata, Journal of Chromatography A, 881 (2000) 425.

[15] G.L. Roberston, D. Swinburne, Journal of Food Science, 46 (1981) 1557.

[16] L.F. Wang, S.C. Park, J.O. Chung, J.H. Baik, S.K. Park, Journal of Food Science, 69 (2004) S301.

[17] H.H. Wijngaard, C. Rößle, N. Brunton, Food Chemistry, 116 (2009) 202.

[18] A. Schieber, P. Hilt, P. Streker, H.-U. Endreß, C. Rentschler, R. Carle, Innovative Food Science & Emerging Technologies, 4 (2003) 99.

[19] A. Del Caro, A. Piga, I. Pinna, P.M. Fenu, M. Agabbio, Journal of Agricultural and Food Chemistry, 52 (2004) 4780.

[20] I. Ignat, I. Volf, V.I. Popa, Food Chemistry, 126 (2011) 1821.

[21] L. Normen, S. Bryngelsson, M. Johnsson, P. Evheden, L. Ellegård, H. Brants, H. Andersson, P. Dutta, Journal of food composition and analysis, 15 (2002) 693.

[22] R.D. Belding, S.M. Blankenship, E. Young, R.B. Leidy, Journal of the american society for horticultural science, 123 (1998) 348.

[23] J. Zhang, Z.-Liu,, Acta Horticulturae Sinica, 5 (2009) 005.

[24] R.D. Belding, S.M. Blankenship, E. Young, R.B. Leidy, Journal of the american society for horticultural science, 123 (1998) 348.

[25] E.-I. Geană, R.E. Ionete, A. Ciocarlan, A. Aricu, A. Fulga, N. Ungur, M. Podogova, D. Nikolaeva, Progress of Cryogenics & Isotopes Separation, 17 (2014).

[26] A.-L. Fanciullino, M. Cercós, C. Dhuique-Mayer, Y. Froelicher, M. Talón, P. Ollitrault, R. Morillon, Journal of Agricultural and Food Chemistry, 56 (2008) 3628.

[27] A.J. Meléndez-Martínez, I.M. Vicario, F.J. Heredia, Journal of Food Composition and Analysis, 20 (2007) 638.

[28] R. Rouseff, L. Raley, H.-J. Hofsommer, Journal of Agricultural and Food Chemistry, 44 (1996) 2176.

[29] L.Q. Zepka, A.Z. Mercadante, Food Chemistry, 117 (2009) 28.

[30] M. Spraul, B. Schütz, P. Rinke, S. Koswig, E. Humpfer, H. Schäfer, M. Mörtter, F. Fang, U.C. Marx, A. Minoja, Nutrients, 1 (2009) 148.

[31] F. Zeng, J. Ou, Y. Huang, Q. Li, G. Xu, Z. Liu, S. Yang, Food Analytical Methods, 8 (2015) 428.

[32] R. Zadernowski, S. Czaplicki, M. Naczk, Food Chemistry, 112 (2009) 685.

CHAPTER 5. CONCLUSIONS

The main conclusions drawn from the studies presented in this Doctoral Thesis can be summarised as follows:

1. The developed UPLC-PDA-MS/MS method allows the determination of up to 27 carotenoids compounds simultaneously. The determination in less than 17 min of epoxycarotenoids (including 5,6 and 5,8 isomers), hydroxycarotenoids and carotenes in freshly made and industrially processed fruit juices has been described for first time.

2. The carotenoid extraction method developed (included saponification step) provided good recoveries and excellent repeatability. Different solvents and solvent mixtures were tested to assess extraction efficiencies on lyophilised material. Methanol provided the highest carotenoid extraction capability.

3. The carotenoid profile of freshly made fruit products is dominated by 5,6-epoxycarotenoids. However, they do not appear in the processed samples where an increase of 5,8-epoxycarotenoids is observed.

4. The UPLC-PDA-MS/MS method developed in this Thesis allows to determine up to 48 chlorophyll derivatives. The simultaneous separation of the chlorophyll derivatives resulting from demetalitation, dephylitation, decarbomethoxylayion, epimerisation and copperisation is achieved using a ternary gradient in less than 13 min.

5. The analysis of chlorophyll derivatives was applied to assess the degree of processing in different teas. The applied method allows the differentiation between common and luxurious commercial teas and among tea varieties.

6. The analysis of chlorophyll derivatives was also applied to study the quantities of pheophytins, pheophorbides and pyro derivatives in processed green fruit and vegetable products. Simultaneously, the developed UPLC-PDA-MS/MS method allowed the detection of food Cu-chlorophylls in various samples.

7. The phenolic profiles of nine processed fruit fibres from the juice industry were determined by UPLC-PDA-FLR-MS/MS, identifying and quantifying 40 phenolic compounds in these fibres. In addition, bound phenolic acids in the residue were measured by HPLC-PDA and also bound

proanthocyanidins in the same residue were determined by a colorimetric assay, both of them after being extracted through hydrolysis.

8. In general, the phenolic profiles of fibres were consistent with those related in the literature for fresh fruit. However, the comparison of fibres with lyophilised peel and flesh from fresh fruit was more complex. The phenolic content and degradation behavior depended on the type of fruit and its processing, as well as on the family of phenolic compounds assessed.

9. Given the health benefits of phenolic compounds, and based on our findings about their phenolic profiles, we conclude that the fibres from fruit and vegetable by-products can provide high added value as ingredients for the food manufacturing industry.

10. Non-volatile primary and secondary metabolites (such as: sugars, sugar alcohols, low molecular weight organic acids, amino acids, fatty acids phytosterols, triterpenes, fatty alcohols, alkanes and carotenoids) were characterised in nine processed fruit fibres from the juice industry. To identify and quantify these metabolites instrumental techniques as GC-MS, GC-FID and UPLC-PDA-MS/MS were applied.

11. The metabolite profiles of fibres were consistent with those related in the literature for fresh fruit and fruit juices. The content of primary metabolites, as well as some secondary metabolites as phytosterols and triterpens was little affected by the process used to obtain fibres. In contrast, the level of carotenoids was highly affected, in terms of content and profile, during the processing.

12. We authenticated monovarietal peach purees and pear juices on the basis of primary metabolite and phenolic compound analysis by ^{1}H-NMR and UPLC-PDA-MS/MS, respectively. After suitable preprocessing, ^{1}H-NMR and chromatographic data was evaluated by PCA, both separately and pooled.

13. The PCA of primary metabolites allowed the discrimination between peach varieties and pear varieties, respectively. But did not allow discriminating between industrially processed and non-processed purees. On the other hand, PCA approach for phenolic compounds discriminated between

varieties in peach purees and pear juices as well as between processed and non-processed peach purees. The combined PCA data for primary metabolites and phenolic compounds improved the separation of clusters in all cases. This approach may help to better control the cultivar authenticity in fruit products. Consequently, it may contribute to the development of a process to achieve commercial products with the characteristic quality of a given cultivar.

APPENDIX

Appendix I. Abbreviations used in this Doctoral Thesis.

3-Caf-qui	3-Caffeoylquinic acid
4-Caf-qui	4-Caffeoylquinic acid
5-Caf-qui	5-Caffeoylquinic acid
5-p-Cou-qui	5-p-Coumaroylquinic acid
ACN	Acetonitrile
AJ	Alejandrina juice
ALA	Alanine
APCI	Atmospheric pressure chemical ionisation
API	Atmospheric pressure ionisation
APPI	Atmospheric pressure photo-ionisation
AQC	6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate
Arb	Hydroquinone β-D-glucopyranoside or Arbutin
ASP	Asparagine
BHT	Dibutylhydroxytoluene or butylated hydroxy toluene
BJ	Blanquilla juice
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
C	Commerical
CA	Citric acid
Cat	(+)-Catechin
Chlid	Chlorophyllide
CJ	Conference juice
Cou-hex	Coumaroyl-hexose
DAGs	Diacylglycerols
DCM	dichloromethane
DEEMM	Diethyl ethoxymethylenemalonate
DMF	Dimethylformamide
DNS	Dansyl chloride
EI	Electron ionisation
ELSD	Evaporative light scattering detector
Epi	(-)-Epicatechin
ESI	Electrospray ionisation
EU	European union
FAB	Fast atom bombardment
FAMEs	Fatty acid methyl esters

FAs	Fatty acids
FB	Frehly blended
FID	Flame ionisation detection
FLD	Fluorescence detector
FMOC	9-Fluorenylmethylchloroformate
FRU	β-D-fructopyranose
GC	Gas chromatography
GCxGC	Two dimensional gas chromatography or comprehensive gas chromatography
HAc	Acetic acid
HFor	Formic acid
HMF	5-(Hydroxymethyl)furfural
HPLC	High performance liquid chromatography
HR-MAS	High-resolution magic angle spinning
iPrOH	Isopropanol
IS	Internal standard
Iso-6"-malgal	Isorhamnetin 3-O-6"-malonygalactoside
Iso-gal	Isorhamnetin-3-O-galactoside
Iso-glu	Isorhamnetin-3-O-glucoside
Iso-malgal	Isorhamnetin 3-O-malonygalactoside
Iso-rob	Isorhamnetin-3-O-robinioside
Iso-rut	Isorhamnetin-3-O-rutinoside
IT	ion trap
K-rut	Kaempferol 3-O-rutinoside
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantitation
M	Miraflores
MA	Malic acid
MAE	Microwave-assisted extraction
MAGs	Monoacylglycerols
MALDI	Matrix assisted laser desorption ionisation
MDG	Millennium developed goal
MeCN	Acetonitrile
MeOH	Methanol
MEOX	Methoxyamine hydrochloride

MES	2-(N-morpholino)ethanesulfonic acid
MRM	Multiple Reaction Monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSTFA	N-Methyl-N-(trimethylsilyl)
MTBE	Methyl-*tert*-butyl ether
MUFA	Monounsaturated fatty acids
NFC	Juice not from concentrate
NIR	Near infrared spectroscopy
NMR	Nuclear magnetic resonance
NP	Normal phase
OPA	O-phthaldialdehyde
PC	Principal component
PCA	Principal component analysis
PDA	Photodiode array detector
PITC	Phenylisothiocianate
PLE	Pressurised liquid extraction
Pro-B1	Procyanidin-B1
Pro-B2	Procyanidin-B2
ProCat-glu	Protocatechuic aldehyde monoglucuronide
PTFE	Polytetrafluoroethylene
PUFA	Polyunsaturated fatty acids
Q	Q-bands
QA	Quinic acid
Q-gal	Quercetin-3-O-galactoside
Q-glu	Quercetin-3-O-glucoside
QQQ	Triple quadrupole
Q-rut	Quercetin-3-O-rutinoside
Q-TOF	Quadrupole-time of flight
RFC	Reconstituted from concentrate
RID	Refractive index detector
RP	Reversed-phase
RSD	Relative standard deviation
S	Soret
SCC	Sodium copper chlorophyllin

SFA	Saturated fatty acids
SFE	Supercritical fluid extraction
SIM	Selected ion monitoring
SL	Spring Lady
SPE	Solid phase etraction
SUCR	Sucrose
TAGs	Triacylglycerols
TAHS	p-N,N,N-trimethylammonioanilyl N'-hydroxysuccinimidyl carbamate iodide
THF	Tetrahydrofuran
TMS	Trimethylsilyl
TPC	Total phenolic content
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
UAE	Ultrasound-assisted extraction
UF	Ultrafiltration
UPLC	Ultra-performance liquid chromatography
UV	Ultraviolet
UV-Vis	Ultraviolet-visible
αGLC	α-glucose
βGLC	β-glucose

Appendix II.

LIST OF PUBLICATIONS.

Antoni Delpino-Rius, Jordi Eras, Francisca Vilaró, Miguel Ángel Cubero, Mercè Balcells, and Ramon Canela-Garayoa**. "Characterisation of phenolic compounds in processed fibers from the juice industry."** *Food Chemistry 172* (2015) 575-584. DOI: 10.1016/j.foodchem.2014.09.071.

Antoni Delpino-Rius, Jordi Eras, Alexis Marsol-Vall, Francisca Vilaró, Mercè Balcells, and Ramon Canela-Garayoa**. "Ultra performance liquid chromatography analysis to study the changes in the carotenoid profile of commercial monovarietal fruit juices."** *Journal of Chromatography A* 1331 (2014) 90-99. DOI: 10.1016/j.chroma.2014.01.044

MANUSCRIPTS SUBMITTED

Characterisation of primary and secondary metabolites in processed fruit fibres from the juice industry. Antoni Delpino-Rius, Jordi Eras, Francisca Vilaró, Miguel Ángel Cubero, Mercè Balcells, and Ramon Canela-Garayoa**.** Submitted to *Food Composition and Analysis.*

A fast and reliable UPLC method to assess the fate of chlorophylls in processed vegetable products.. Antoni Delpino-Rius, Diana G. Cosovanu, Mercè Balcells, Jordi Eras, Ramon Canela-Garayoa. Submitted to *Journal of Chromatography A.*

Combined analysis of primary metabolites and phenolic compounds to authenticate commercial monovarietal peach purees and pear juices. Antoni Delpino-Rius, Jordi Eras, Mercè Balcells, and Ramon Canela-Garayoa**.** Submitted to *Food Control.*

CONFERENCES

Chlorophyll-derived compounds analysis in fruit and vegetable products by UPLC-PDA-MS/MS. Delpino-Rius, A.; D.G. Cosovanu; Balcells, M.; Eras, J.; Canela, R. 14th Instrumental Analysis Conference (JAI), 2014. Barcelona (Spain). **Poster.**

Development of advanced analytical methods in fruit safety. Delpino-Rius; A Marsol, A. Chemical Safety. Seminar of the Tempus Project "Improving Academia-Industry Link in Food Safety, 2013. Lleida (Spain). **Oral communication.**

Characterization of antioxidant compounds in fruit fibers. Delpino-Rius, A.; Eras, J.; Balcells, M.; Canela, R. 6th International Symposium on Recent Advances in Food Analysis (RAFA), 2013 Prague (Czech Republic). **Poster.**

Metabolome characterization in fruit and fruit fibers with hyphenated mass spectrometry. del Pino, A.; Marsol, A.; Balcells, M.; Llovera, M.; Canela, R.; Eras, J. Jornada "Avenços de l'espectrometria de masses per al control de la seguretat i l'autenticitat dels aliments", 2013. Barcelona (Spain). **Poster.**

Accurate and reliable determination of carotenoids in aple fruits and juices by HPLC-PDA. Delpino-Rius, A.; Eras, J.; Balcells, M.; Canela, R. XII Scientific Meeting of the Spanish Society of Chromatography and related techniques, 2012. Tarragona (Spain). **Poster**.