Gas chromatography-mass spectrometry for the analysis of metabolomic compounds in agrifood products. New methods and applications

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Gas Chromatography-Mass Spectrometry for the Analysis of Metabolomic Compounds in Agrifood Products. New Methods and Applications.

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SUMMARY

This Doctoral Thesis focuses on the development of novel gas chromatography coupled to mass spectrometry (GC-MS) techniques and the application of some existing methods to the analysis of fruit and fruit-derived samples. The thesis is divided in three parts attending the approaches studied.

Initially, a comprehensive two-dimensional gas chromatography (GC×GC-MS) method was developed by testing several column configurations to analyse apples and peaches. Results showed the semi-polar/non-polar configuration as the most suitable for this purpose. Moreover, the composition between fruits (apple and peach) was significantly different while cultivars within fruits showed very similar results.

In the second part of the Thesis, three new methods based on injection-port derivatization were developed. The first consisted on a targeted analysis of 17 glycosylated and non-glycosylated polyphenols in fruit and fruit juice samples. With the aid of multiple reaction monitoring (MRM) low LOD and adequate performance parameters were achieved. This method required the introduction of a clean-up step prior to the analysis of the extract due to the high content of other polar compounds in the extracts, which led to important matrix effects. The second method was devoted to the analysis of HMF and patulin, two compounds used as markers of quality in the fruit juice industry. Injection-port derivatization allowed direct analysis of the organic extract into the GC. The methodology provided LOD and LOQ compliant with current legal levels for these two compounds in fruit juice products. In addition, the simplicity and ease of the method showed to be very useful for juice samples analysis. The last method developed in this part was focused on the free lipophilic fraction of fruit juices. In this case, a dispersive liquid-liquid microextraction (DLLME) preceded in-port derivatization. DLLME was optimized employing a full-factorial experimental design to determine the most significant parameters. Given that an in-port compatible solvent was used, the extract was directly analysed. The developed method was fully validated giving an adequate performance.

The third part was devoted to the analysis of volatile and semi-volatile compounds in several fruit-derived products, namely fruit fibres deriving from the juice industry and four samples of peach juices consisting in two varieties (yellow and red-fleshed) and two distinct processing procedures for each variety (freshly blended and commercial). In the first study solid-phase microextraction (SPME) was used as routine technique to sample the volatile fraction. In addition, enantiomeric analysis of the fruit fibre samples revealed variation of the enantiomeric ratio of the asymmetric monoterpene alcohols present in these samples. This was probably caused by the thermal treatment of the fibre during its production. Finally, the last study was performed using a PDMS-coated stir bar sorptive extraction (SBSE). The volatile and semi-volatile fraction were analysed by direct immersion of the stir bar followed by thermal desorption and GC-MS analysis. The analytical data obtained were analysed using conventional chemometric tools showing a differentiation between samples by means of 14 selected variables.

RESUM

Aquesta Tesi Doctoral se centra en el desenvolupament de nous mètodes de cromatografia de gasos acoblada a tècniques d'espectrometria de masses (GC-MS) i a l'aplicació d'alguns mètodes ja existents a l'anàlisi de mostres de fruites i derivats. La tesi es divideix en tres parts segons els enfocaments estudiats.

Inicialment, es va desenvolupar un mètode de cromatografia de gasos bidimensional comprensiva (GC×GC-MS) en la qual es van provar diverses configuracions de columnes. Els resultats van mostrar que la configuració semipolar/no-polar és la més adequada. A més, la composició entre fruits (poma i préssec) va ser significativament diferent, mentre que les varietats d'un mateix fruit van ser molt similars.

A la segona part de la Tesi, es van desenvolupar tres nous mètodes basats en la derivatització al port d'injecció. La primera va consistir en l'anàlisi selectiu de 17 polifenols glicosilats i no glicosilats en mostres de fruita i suc de fruita. Amb l'ajuda del monitoratge de reacció múltiple (MRM) es van obtenir baixos LOD i paràmetres de rendiment adequats. Aquest mètode va necessitar d'una etapa de neteja abans de l'anàlisi de l'extracte causat per l'alt contingut d'altres compostos polars, que donaven lloc a importants efectes matriu. El segon mètode es va destinar a l'anàlisi de HMF i patulina, dos compostos utilitzats com a marcadors de qualitat en la indústria dels sucs de fruites. La derivatització per injecció-port va permetre l'anàlisi directa de l'extracte orgànic en el GC. La metodologia va proporcionar LOD i LOQ compatibles amb els actuals nivells legals per a aquests dos compostos en sucs. L'últim mètode desenvolupat en aquesta part es va centrar en la fracció lipofílica lliure de sucs de fruita. En aquest cas, una microextracció líquid-líquid dispersiva (DLLME) va precedir a la derivatització en el port. La DLLME va ser optimitzada emprant un disseny experimental factorial complet per determinar els paràmetres més significatius. Atès que es va utilitzar un dissolvent compatible, l'extracte es va analitzar directament. El mètode desenvolupat va ser completament validat donant un rendiment adequat.

La tercera part es va centrar en l'anàlisi dels compostos volàtils i semi-volàtils de diversos derivats de la fruita, a saber, fibres de fruita derivades de la indústria dels sucs i quatre mostres de sucs de préssec consistents en dues varietats (groc i vermell) i dos procediments d'elaboració per a cada varietat (recentment liquat i comercial). En el primer estudi es va utilitzar SPME com a tècnica per mostrejar la fracció volàtil. A més, l'anàlisi enantiomèric de les mostres de fibra de fruita va revelar una variació en els alcohols monoterpènics asimètrics. Això probablement va ser causat pel tractament tèrmic de la fibra durant la seva producció. Finalment, l'últim estudi es va realitzar utilitzant una extracció sorptiva amb barra d'agitació revestida amb PDMS (SBSE). La fracció volàtil i semi-volàtil es va analitzar per immersió directa seguida de desorció tèrmica i anàlisi per GC-MS. Les dades analítiques obtingudes es van analitzar utilitzant eines quimiomètriques convencionals mostrant una diferenciació entre mostres mitjançant 14 variables seleccionades.

RESUMEN

Esta Tesis Doctoral se centra en el desarrollo de nuevos métodos de cromatografía de gases acoplada a técnicas de espectrometría de masas (GC-MS) y a la aplicación de algunos métodos existentes al análisis de muestras de frutas y derivados. La tesis se divide en tres partes según los enfoques estudiados.

Inicialmente, se desarrolló un método de cromatografía de gases bidimensional comprensiva (GC×GC-MS) en la que se probaron varias configuraciones de columnas. Los resultados mostraron que la configuración semipolar/no-polar es la más adecuada. Además, la composición entre frutos (manzana y melocotón) fue significativamente diferente, mientras que las variedades de un mismo fruto resultaron muy similares.

En la segunda parte de la Tesis, se desarrollaron tres nuevos métodos basados en la derivatización en el puerto de inyección. La primera consistió en un análisis selectivo de 17 polifenoles glicosilados y no glicosilados en muestras de fruta y zumo de fruta. Con la ayuda de la monitorización de reacción múltiple (MRM) se obtuvieron bajos LOD y parámetros de rendimiento adecuados. Este método requirió de una etapa de limpieza antes del análisis del extracto debido al alto contenido de otros compuestos polares, que daban lugar a importantes efectos matriz. El segundo método se dedicó al análisis de HMF y patulina, dos compuestos utilizados como marcadores de calidad en la industria del zumo de frutas. La derivatización por inyección-puerto permitió el análisis directo del extracto orgánico en el GC. La metodología proporcionó LOD y LOQ compatibles con los actuales niveles legales para estos dos compuestos en zumos. El último método desarrollado en esta parte se centró en la fracción lipofílica libre de zumos de fruta. En este caso, una microextracción líquido-líquido dispersiva (DLLME) precedió a la derivatización en el puerto. La DLLME fue optimizada empleando un diseño experimental factorial completo para determinar los parámetros más significativos. Dado que se utilizó un disolvente compatible, el extracto se analizó directamente. El método desarrollado fue completamente validado dando un rendimiento adecuado.

La tercera parte se dedicó al análisis de los compuestos volátiles y semi-volátiles de varios derivados de la fruta, a saber, fibras de fruta derivadas de la industria de los zumos y cuatro muestras de zumos de melocotón consistentes en dos variedades (amarillo y rojo) y dos procedimientos de elaboración para cada variedad (recién licuado y comercial). En el primer estudio se utilizó SPME como técnica para muestrear la fracción volátil. Además, el análisis enantiomérico de las muestras de fibra de fruta reveló una variación en los alcoholes monoterpénicos asimétricos. Esto probablemente fue causado por el tratamiento térmico de la fibra durante su producción. Finalmente, el último estudio se realizó utilizando una extracción sorptiva con barra de agitación revestida con PDMS (SBSE). La fracción volátil y semi-volátil se analizó por inmersión directa seguida de desorción térmica y análisis por GC-MS. Los datos analíticos obtenidos se analizaron utilizando herramientas quimiométricas convencionales mostrando una diferenciación entre muestras mediante 14 variables seleccionadas.

ACRONYMS

1D	One-dimensional
2D	Two-dimensional
2D-GC	Heart-cutting two-dimensional gas chromatography
AMU	Atomic mass units
ANOVA	Analysis of variance
BSA	*N,O*-Bis(trimethylsilyl)acetamide
BSTFA	*N,O*-Bis(trimethylsilyl)trifluoroacetamide
C18	Octadecyl carbon chain
CAR	Carboxen
CIS	Cooled injection system
CTMS	Trimethylchlorosilane
CW	Carbowax
D	Dynamic
DAD	Diode array detector
DAG	Diacylglycerol
DF	Dietary fibre
DI	Direct immersion
diEt-CD	2,3-di-*O*-ethyl-6-*O*-*tert*-butyldimethylsilyl-β-cyclodextrin
DLLME	Dispersive liquid-liquid microextraction
DVB	Divinylbenzene
EF	Enrichment factor
EI	Electron ionization
ER	Enantiomeric ratio
Es	Enantioselective
ESI	Electrospray ionization
EtOAc	Ethyl acetate
FID	Flame ionization detector
FRAP	Ferric reducing/ antioxidant power
GC	Gas chromatography
GCxGC	Comprehensive two-dimensional gas chromatography
GMD	Golm metabolome database

HAcO	Acetic acid
HCC	High concentration capacity
HF	Hollow-fibre
HMDS	Hexamethyldisilazane
HMF	5-(Hydroxymethyl)furfural
HPLC	High performance liquid chromatography
HS	Headspace
ID	Inner diameter
IL	Ionic liquid
IS	Internal standard
IT	In-tube
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LPME	Liquid-phase microextraction
MAG	Monoacylglycerol
ME	Matrix effects
MeOH	Methanol
MEOX	Methoxylamine hydrochloride
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSTA	*N*-Methyl-*N*-trimethylsilylacetamide
MSTFA	*N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide
ODMS	O-dimethylsilyl
OTMS	O-trimethylsilyl
P&T	Purge-and-trap
PA	Polyacrylate
PCA	Principal component analysis
PDA	Photodiode array
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
Pentyl-CD	2,6-di-methyl-3-*O*-pentyl-β-cyclodextrin
PTFE	Polytetrafluoroethylene

PTV	Programmable temperature vaporizing
Q	Quadrupole
QqQ	Triple quadrupole
QuEChERS	Quick, easy, cheap, effective, rugged, and safe
RI	Retention index
RSD	Relative standard deviation
RTI	Retention time index
S	Static
S/N	Signal-to-noise ratio
SBSE	Stir bar sorptive extraction
SD	Standard deviation
SDME	Single-drop microextraction
SFE	Supercritical fluid extraction
SIM	Selected ion monitoring
SLDA	Stepwise linear discriminant analysis
SLM	Supported liquid membrane
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
TAA	Tetraalkylammonium
TAC	Total antioxidant content
TAG	Triacylglycerol
TCD	Thermal conductivity detector
TD	Thermal desorption
TDU	Thermal desorption unit
TIC	Total ion current
TMS	Trimethylsilyl
TMSDEA	*N*-Trimethylsilyldiethylamine
TMSIm	Trimethylsilylimidazole
TOF	Time of flight
UV	Ultraviolet

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CHAPTER 1. INTRODUCTION

Fruits and fruit-derived products, especially juices, are important commodities in current European life-style. In this regard, mean daily fruit consumption in Europe was 167.62 g/capita/day [1]. According to the European Fruit Juice association market report of 2015, 9.702 million litres of fruit juice were consumed in Europe in 2014 being orange and apple juices the most consumed flavours, representing 70% of total juice consumption (Figure 1) [2].

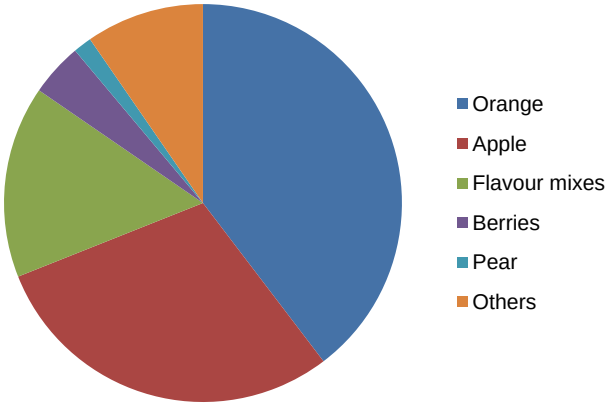

Figure 1: Juice consumption distribution between fruits in Europe [2].

Fruit production in Europe accounted for 32.5 million mT in 2012 [1]. Regarding to the European producers of fruits, the climate of southern EU may favour citrus fruits (the vast majority of citrus fruits (59.8%) are produced in Spain), but the North and the Centre are home to many varieties of the Rosaceae –apples (14 million tonnes of apples were produced in the EU-28 in 2014), pears, plums, strawberries, and raspberries. Nevertheless, this genus is still more commonly found in Southern EU especially peaches, apricots, and cherries. Vitaceae genus, mainly composed by grapes, while they are more prolific in southern countries they can also be found in the whole continent [3]. Although Europe is an important fruit producer, as shown in Figure 2, at a worldwide scale Asia is the continent with the highest production, followed by the Americas and Europe, which has global fruit production very similar to Africa [4].

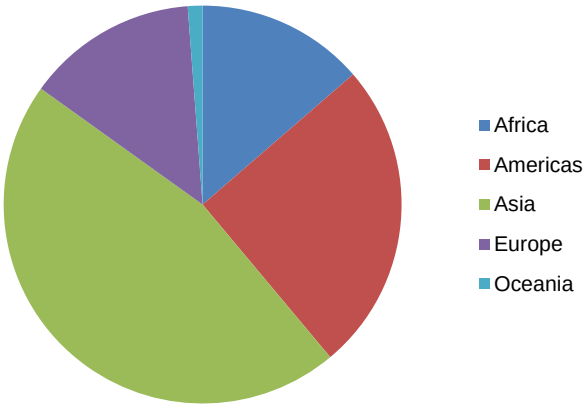

Figure 2: Total fruit production by continent [4].

As mentioned before, fruit production is greatly dependent on the climate of a certain geographical region. For this reason there is an important trade between countries Spain being the Europe's biggest exporter of fresh fruit. With an export volume of almost 7.2 million, this accounts for 34% of Europe's fresh fruit exportations. Other EU exporter countries are Italy and the Netherlands. Major destinations of exportations are Germany, the UK, and France (around 44%). Furthermore, outside the EU, fresh fruit and vegetables are mainly exported to Russia, Belarus, Ukraine, Switzerland, and Norway [5].

Fruits are essential ingredients for a balanced diet as they are natural sources of various vitamins, minerals, fibres, and phytochemicals; the last being responsible, in part, for the antioxidant properties of fruits and foods of plant origin. Manufacturing processes can affect nutritional properties of some foods. Otherwise, some nutrients and bioactive compounds, which are naturally present in fruits, may undergo transformations during food processing that decrease neither their nutritional value nor bioactive value but may increase it by favouring their absorption and metabolism in the human body [6].

Additionally to fruit consumption as raw products, fruit processing plants produce a wide variety of fruit-derived products including concentrate juices, cloudy juices, clarified juices, and purees. Concentrate juice production involves concentration by heating under vacuum to remove water, and then cooling to around 13 ºC. With this procedure about two thirds of the water in a juice are removed [7]. Cloudy juices derive from naturally squeezed fruits. No water is intentionally added or removed and processing is designed to retain as much as possible the character of the raw fruit. Clarified juices are obtained from naturally squeezed fruits juices, which are submitted to ultrafiltration to remove the fine suspended particles of pulp. Purees are aimed to retain all of the juice and a large

proportion of the fibrous matter naturally found in the fruit. Consequently, only seeds or stones are removed and, sometimes, the excess of insoluble fibres. Hence, fruit fibres appear as a by-product of the fruit juice industry. Most of fibre production processes include a sieve stage (or multiple sieve stages) to ensure the final product is sufficiently smooth and homogenous. Fruit subproducts of the juice production are washed, milled, and dried to obtain a powder which can be valorised to act as a bulking agent, contributing to normalizing intestinal motility and preventing diverticular disease [8]. Considerable attention has also been paid on the incidence of a number of non-infectious diseases common in civilised societies, such as coronary heart disease, which could be attributed to a low dietary fibre (DF) intake. Some types of DF may also be important in reducing colonic cancer, in lowering serum cholesterol levels and in preventing hyperglycaemias in diabetic patients [9].

There is a considerable research activity dealing with the analysis of several classes of metabolomic compounds in fruit and fruit-derived samples. These analyses are mainly conducted using separative techniques like HPLC and GC coupled with mass spectrometry (MS). Among the vast amount of publications employing the above mentioned techniques we find: analysis of volatiles either using SPME in HS [10-14] or in direct immersion mode (DI) [15] or SBSE [16-18], analysis of bioactive compounds like polyphenols [19-22] and carotenoids [23-25], determination of lipophilic compounds [26-29], sugars, organic acids and aminoacids [22, 30-32], and toxins [33-35]. Consequently, the analysis of several classes of phytochemicals in fruits and fruit-derived products has become a matter of interest and new advanced analytical methodologies have emerged. As stated by Belitz *et al.* [36] a comprehensive evaluation of foods requires that analytical techniques keep pace with the available technology. As a result, a major objective in food chemistry is concerned with the application and continual development of analytical methods.

1.1. Metabolomic compounds in fruit and fruit-derived products

The analysis of metabolomic compounds present in food, as a whole, but also in the subclass comprised by fruits and fruit-derived products has been a matter of interest in modern analytical approaches as some of these compounds can be very useful to assess food quality and geographical origin, food traceability, detection of pathogens and toxins, and detection of food allergens [37]. Obviously, such matrices include several different classes of chemical compounds with very different properties as food chemistry, unlike other branches of chemistry which are concerned either with particular classes of compounds or particular methods, is a subject which, both in terms of the actual chemistry and the methods involved, has a very broad field to cover [36]. Within the scope of this Thesis, the analysed compounds have been divided in two groups. A first group including disaccharides, polyphenols, lipophilic compounds, and patulin and 5-hydroxymethylfurfural, most of them high boiling compounds, and a second group composed by volatile and semi-volatile compounds. This classification has been done considering they require different approaches in sample preparation and analysis.

1.1.1. High boiling compounds

Compounds containing hydrogen atoms bound to electronegative elements such as oxygen, nitrogen, sulphur or phosphorous are considered as polar compounds, which due to their polar nature require high temperatures to generate sufficient vapour pressure to achieve a phase change to gas [38]. Hence, these compounds will require specific analytical treatment for their analysis by gas chromatography (GC). Among the vast number of existing high boiling compounds in fruit-derived samples, in this Thesis we divided the analysed compounds in four well-defined groups according to their chemical nature.

1.1.1.1. Carbohydrates. Disaccharides

Carbohydrates are primary metabolites, i.e. compounds directly involved in normal growth, development, and reproduction which represent one of the basic nutrients and are quantitatively the most important source of energy [36]. Traditionally, they are divided into monosaccharides, (glucose, fructose, galactose…) oligosaccharides (including disaccharides and trisaccharides) and polysaccharides (starch, cellulose and pectin), which are formed through condensation of monosaccharide units. Monosaccharides are polyhydroxy-aldehydes (aldoses) or polyhydroxyketones (ketoses). Disaccharides, which derive from a combination of two monosaccharide units and with different types of linkage result in a high number of isomers, which in its turn may lead to complex chromatograms. Figure 3 shows the structure of some of the naturally occurring disaccharides. The importance of disaccharides in fruit is especially noticeable in the case of sucrose, which is the most abundant sweetener in fruits, and in the case of α,α-

trehalose, which is considered to be an abiotic stress biomarker [39, 40] and a preservation agent [41, 42].

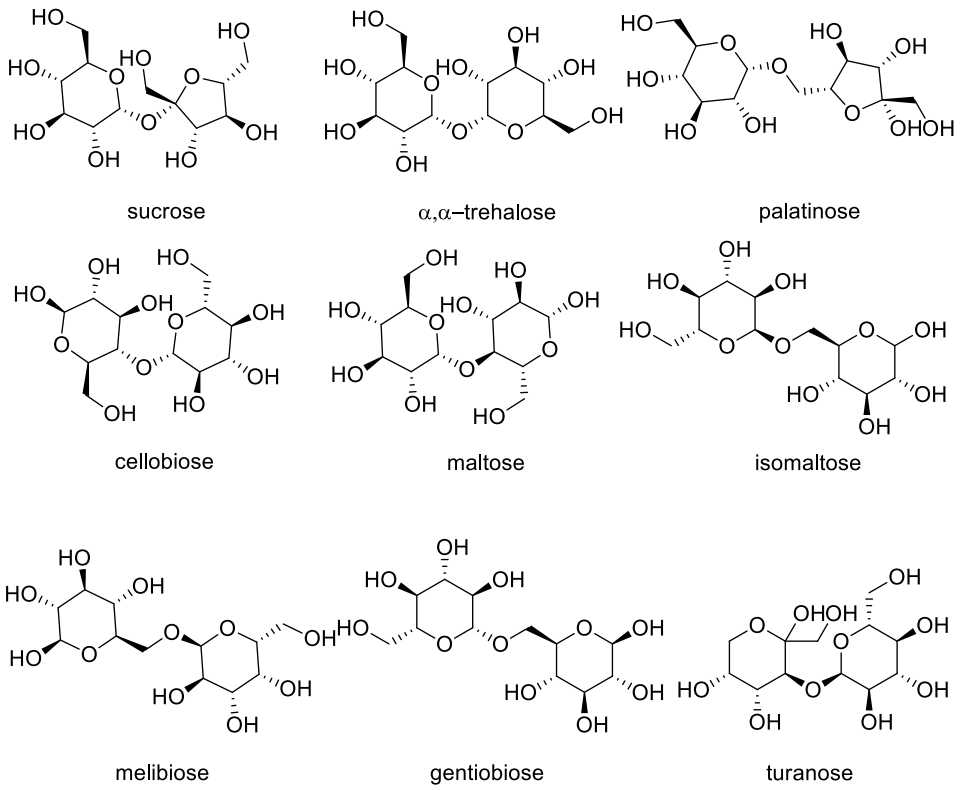

Figure 3: Chemical structure of some of the disaccharides analysed.

In 2000, Roessner *et al.* [43] described the procedure to analyse the polar fraction of vegetable samples, which includes disaccharides. This article details a GC methodology employed in many subsequent publications focused on the analysis of sugars, aminoacids and organic acids in vegetable samples. This procedure involves extraction of the solid sample with a mixture of polar solvents, water and methanol, followed by drying *in vacuo* of the extract and derivatization with methoxyamine hydrochloride in pyridine (MEOX) and MSTFA. After derivatization, samples are directly analysed by GC-MS. In this procedure, MEOX is employed to suppress the free anomeric centre by combining with the carbonyl group of reducing disaccharides. Therefore, only

two derivatives, *syn* (E) and *anti* (Z), are formed for each reducing disaccharide [44] (Figure 4) while if MSTFA was directly used partial derivatization of the carbonyl group would interfere the chromatographic analysis. This methodology with some modifications has been widely employed to analyse sugars [22, 45-47], and other polar compounds like polyphenols in fruit samples [22, 48].

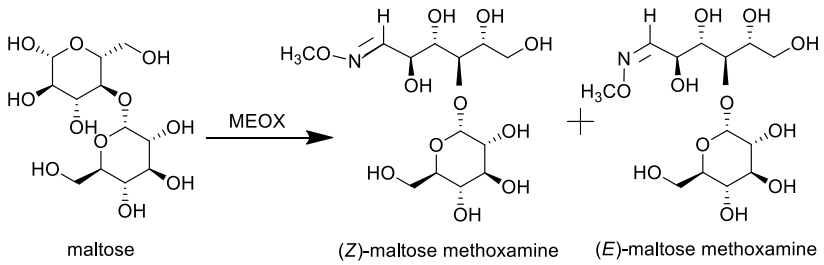

Figure 4: Methoxymation reaction of a reducing disaccharide.

Furthermore, due to the complexity of some samples like honey comprehensive two-dimensional gas chromatography (GC×GC) has also been employed as a more powerful separation technique [49].

1.1.1.2. Polyphenols

Phenolic compounds are defined by the presence of at least one aromatic ring. Polyphenols are phenolic compounds presenting hydroxyl substituents, including their functional derivative (e.g. esters and glycosides) [50]. Polyphenols are considered to be natural antioxidants due to their capacity to react with free radicals (scavenging) [51]. Most of the antioxidants are phenolic compounds which can be grouped into: tocopherols, flavonoids, phenolic acids, cinnamic acid derivatives, lignin, stilbenes, tannins and coumarins [19]. Among them, flavonoids are the main bioactives found in fruit, with over 5000 identified to date [52]. Structurally, flavonoids are formed by a C_6-C_3-C_6 carbon skeleton of two aromatic rings linked by a three-carbon chain which is condensed to form a pyran or a furan ring [53]. Flavonoids can be linked to one or more molecules of monosaccharide giving a flavonoid glycoside. Flavonoids not linked to a monosaccharide are known as flavonoid aglycones. Figure 5 shows the main subclasses of flavonoids: flavonols, flavones, isoflavones, flavanols, anthocyanidins, and chalcones. Flavonols are the most common flavonoids in fruits, either in their glycosylated and non-glycosylated

forms (e.g. quercetin or kaempferol). Flavones are generally found in glyosidic form and are typical compounds of citrus fruits. Isoflavones are principally found in leguminous plants. Flavanols are also typical compounds of fruits both in monomeric (catechin and epicatechin) and polymeric (proanthocyanidins) forms. Anthocyanidins are common plant pigments conferring red, blue, purple, violet and intermediate red purple to berries and other fruits [54]. Their structure is characterised by containing the flavylium ion or 2-phenylchromenylium, which is an oxonium ion (benzopyrylium). Chloride is usually the counterion of the flavylium cation. This positive charge is a remarkable difference between anthocyanidins and the other flavonoids.

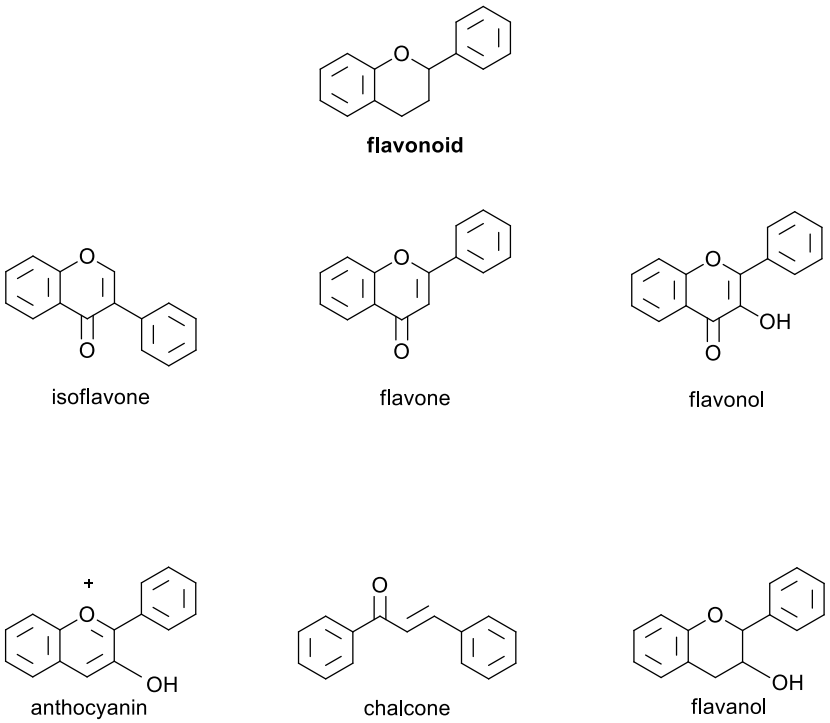

Figure 5: Basic chemical structures of the main classes of flavonoids.

The recognised importance of antioxidants in fruits has led to several methods for measuring their content. These methodologies can be clearly divided between methodologies devoted to the analysis of total antioxidant content (TAC) and those aiming to identify and quantify a specific family or type of phenolic compounds. In regards of the

methodologies for measuring TAC two popular widely used methods are Ferric Reducing/ Antioxidant Power (FRAP) [55] and Folin-Cicolteau [56]. On the other hand, methods focused on the analysis of individual polyphenols are, almost exclusively, based on chromatographic methods. Thereby, HPLC is the most widely used technique for separation purposes employing reversed-phase columns coupled to UV-vis detectors, such as photodiode array (PDA) [57-60]. MS has also been used as a technique very useful, initially for identification purposes and complementary to PDA [57, 59, 60] but has also achieved popularity for quantitation due to the good performance parameters obtained with electrospray ionization (ESI) source combined with triple quadrupole detectors [61, 62]. Although HPLC is generally the technique of choice in the analysis of polyphenols in fruit samples, there have been several attempts to perform this analysis employing GC, specially concerning the analysis of polyphenol aglycones employing GC-MS with previous derivatization to increase their volatility and thermal stability [63-65].

1.1.1.3. Lipophilic compounds

Lipids in fruit play important roles and serve as structural elements (components of biomembranes in cells) or as storage components (in fruits such as olive and avocado). The denomination of lipids comprehends a series of compounds including fatty acids, diacyl and triacylglicerols, phospholipids, galactolipids, terpenes, sterols, and waxes. Phospholipids, diacylglicerols, and sterols are major components of biomembranes. Besides, other metabolites such as phosphatidic acid, free fatty acids, and diacylglicerol may be present in membranes in variable amounts [66].

Within the scope of this Thesis, some of the compounds present in the lipid fraction of fruit juices have been addressed. In this regard, this group of chemical compounds includes fatty acids, fatty alcohols, sterols, and triterpenes. Figure 6 shows the chemical structure of the compounds under study. Fatty acids are primary metabolites, which consist of a carboxylate group attached to a long hydrocarbon chain. This chain is present in fruit samples in saturated and/or unsaturated forms. Fatty alcohols are long chain alcohols; usually primary alcohols having an even number of carbon atoms. In most of vegetable samples they are present at low concentration [67, 68]. Phytosterols are steroid compounds which occur in plants and vary only in carbon side chains and/or presence or absence of a double bond, they are known to have serum cholesterol lowering effects [69, 70] and immune modulatory activity [71]; for this reason, they have been widely studied in vegetables and fruits [27]. Reported phytosterol data for some plant foods and vegetable oils have shown that nuts and oils contain higher levels (≥1%) of sterols than fruits and vegetables [27]. The compositional distributions of phytosterols in certain vegetable oils have been used for their identification, although their presence as minor compounds [72]. Hence, phytosterols and other non-saponifiable compounds in oils are often used as markers for the assessment of adulterated oils [73-76]. Regarding triterpenes they consist of a pentacyclic structure of six-isoprene units, and although their

biological function and possible benefits for humans are still under consideration some antihypertensive, antiatherosclerotic and antioxidant effects have been pointed [77].

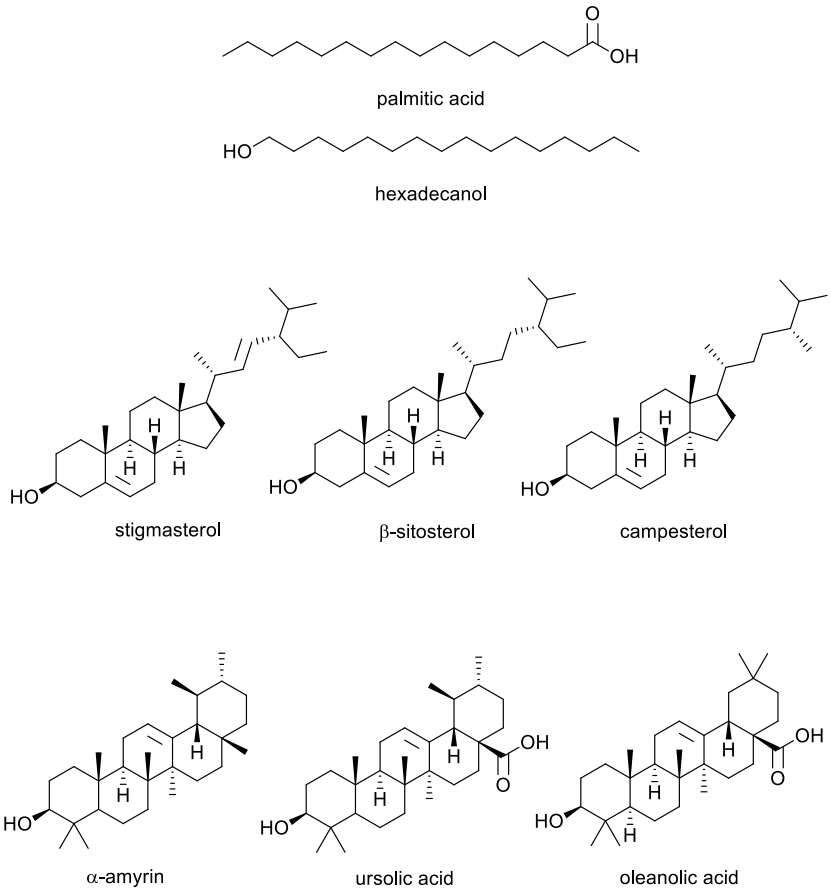

Figure 6: Chemical structure of the lipophilic compounds analysed.

The analysis of lipophilic compounds from vegetable samples is usually preceded by an extraction with organic solvents such as chloroform-methanol [78], hexane [79-81], dichloromethane [82, 83] or acetone [84] followed by saponification and chromatographic purification for obtaining enriched total sterols. Alternatively, the plant samples can be extracted by supercritical fluid extraction (SFE) using supercritical carbon dioxide (CO_2) to obtain total lipid extracts [85-87]. Depending on the samples, saponification is also employed to release the fatty acids present in esters linked to

glycerol (glycerolipids defined as mono- (MAG_S), di-(DAG_S), and triacylglycerols (TAG_S) and their derivatives). The same phenomena may occur for phytosterols.

GC has traditionally been the technique of choice for the analysis of fatty acids, fatty alcohols, phytosterols, and triterpenes. Although the separation of these compounds is likely without derivatization, their analytical performance would be fairly reduced compared with their corresponding derivatives. For these reasons, prior to analysis of the extracts, derivatization is required to enhance chromatographic performance of the compounds due to the presence of polar groups, namely -OH. Hence, available literature reports formation of methyl esters employing acid catalyzers [88, 89] such as, BF_3 [90], HCl [91] or CTMS [92] for fatty acids analysis, while trimethylsilylation is the technique of choice for fatty alcohols [93], phytosterols [94, 95] and triterpenes [96, 97], although acetylation has also been employed in sterol analysis [98].

Compared with GC, HPLC offers the advantage of operating under milder column temperatures, and under non-destructive detection conditions. However, the high lipophilicity of the compounds under study could make sample processing and chromatography difficult.

1.1.1.4. Patulin and 5-hydroxymethylfurfural (HMF)

Patulin (4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one) is a mycotoxin produced by approximately 60 species of microorganisms, including *Aspergillus, Penicillium, Mucor,* and *Fusarium* [99-101]. Some of these microorganisms are responsible for the rotting of fruits like apples, pears, and cherries. Consequently, the presence of patulin in fruit derivatives is an indicator of the quality of the feedstock used in the manufacturing process [102]. The toxicity of patulin has been reviewed in several reports [103]. However, no general consensus has been reached about its true degree of toxicity. In any case, government agencies in the European Union have regulated the following maximum patulin concentration in food products intended for infants and young children: 50 μg/kg in juices; 25 μg/kg in solid apple products; and 10 μg/kg in apple products [104].

5-Hydroxymethylfurfural (HMF) is one of the main products of the Maillard reaction, which may occur during food processing and storage, particularly at high temperatures in carbohydrate-rich products. Moreover, HMF can also be produced during the acid-catalyzed dehydration of hexoses via ring opening followed by 1,2 enolysation and water elimination [105] or by glucosamine hydrolysis [106]. Although HMF is not present in fresh fruits it appears naturally in products in which water coexists with monosaccharides in acid medium, such as balsamic vinegar and fruit juice [107]. In juice industry, HMF is considered as an indicator of the production temperature and storage time as its formation by chemical reaction is supposed to increase with time and temperature.

The analysis of these two compounds is of major importance in fruit juice producing industries as they generally employ as feedstock fruits that are not suitable for direct consume as raw products. In this regard, several research has been conducted to accurately determine this two compounds employing HPLC using a diode array (PDA) [102, 108-110] or a MS detector [111-114] or GC with derivatization to obtain their trimethylsilyl ether, acetate or chloroacetate derivatives using either GC-FID [33, 115] or GC-MS in SIM mode, the latter allowing improved selectivity in complex matrices [116-120]. Methodologies employing GC without derivatization have also been described [116, 121], although they may lead to a diminishment in the analytical performance for patulin.

1.1.2. Volatile and semi-volatile compounds

Volatile compounds are considered those that do not require any derivatization process to be analysed by GC with good chromatographic behaviour. In that sense, this heterogeneous category includes volatile and semi-volatile compounds. Within the frame of this Thesis, Chapter 3 includes the analysis of these compounds in diverse fruit-derived products. Volatiles directly affect the sensorial quality of fresh and processed fruit products, the aroma of which is formed by a complex group of chemical substances (e.g., aldehydes, alcohols, ketones, esters, lactones, and terpenes). These substances are perceived by the odour receptor sites of the olfactory tissue of the nasal cavity. It is well known that the concentration of these volatile compounds is generally low (µg/L) which gives importance to the so-called threshold value (recognition threshold) i.e. the lowest concentration of a compound that is just enough for the recognition of its odour [36]. Aroma composition can be affected by a number of agronomic (variety, climatological conditions, ripening stage) [122-124]; and technological (harvest, post-harvest treatments, storage and processing conditions) factors [125-129]. In the literature a vast number of research papers can be found dealing with the analysis of aroma compounds in fruit samples. Analytical techniques can be divided between dynamic (D-HS) and static headspace (S-HS). In D-HS sampling, the volatile fraction is recovered from the gaseous flow stream stripped *through* (purge-and-trap) (P&T) approach) or *over* (dynamic approach) the matrix onto a suitable trapping system (e.g., cold trap) or medium (a sorbent, an adsorbent, or a specific reagent or solvent for a specific class or classes of compounds). The sampled volatiles are usually released either online by thermal desorption or vaporization (after cryotrapping) directly to the GC or GC-MS system or, more seldom, off-line by solvent elution from the trap. In S-HS sampling, the liquid or solid sample sealed in the headspace vial is equilibrated, at a given temperature for a suitable time; at equilibrium, an aliquot of the resulting vapour phase is transferred to the GC system for analysis [130].

Recent applications show that high concentration capacity (HCC) techniques have been the techniques of choice when performing HS analysis of volatiles in the last years. In this regard, HS-SPME [131] has been used to differentiate between varieties in peach [13], apple [10, 132], raspberry [133] or fruit juices [11]; and to assess storage

conditions [134-137]. Moreover, some applications employing SBSE for berries and peaches have also been described [138, 139].

On the other hand, semi-volatile compounds such as long-chain alkanes or long-chain aldehydes can also be analysed by GC without derivatization although when HS analysis of the samples is carried out the extraction phase presents very low enrichment of these compounds due to their lower vapour pressure. Hence, to achieve a quantitative extraction of semi-volatile compounds direct immersion in the samples is recommended. Although SPME ideal applications deal with HS analysis, some research in DI is also reported such as the analysis of pesticides in lettuce [140]. In addition, to broaden the applicability of SPME, new developments in fibre coatings has been conducted employing sol-gel technology [141], imprinted polymers [142] or ionic liquids [143]. Nevertheless, SBSE was a technique especially conceived for DI analysis due to its robustness and mechanical resistance, which makes this technique very useful for DI sampling of liquid matrices.

1.2. Analytical procedure

The analytical procedure employed in this Thesis is described in the three following sections. The first gives an overview of the extraction techniques employed. The second details derivatization as an important tool in some of the analysis performed. Finally, the chromatographic techniques used are briefly described.

1.2.1. Extraction techniques

Sample preparation is the basic and most crucial step to succeed in any analytical method. To achieve this, three main objectives have to be accomplished: 1) sample matrix simplification and/or replacement, 2) analyte enhancement or concentration, and 3) sample clean-up [66]. Classical sample pre-treatment techniques such as solid-liquid, liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are slow and labour intensive. Often extensive amounts of hazardous organic solvents are used. The main disadvantage of LLE is the necessity of using large amounts of high purity solvents and their subsequent evaporation is an inevitable step in obtaining significant pre-concentration. Thus, this technique is both expensive and environmentally unfriendly [144]. Hence, since 1990's as a result of an increasing concern on the use of organic solvents, which implies production of wastes hazardous for the environment, a broad spectrum of microextraction techniques reducing or even eliminating the use of solvents have appeared.

1.2.1.1. Liquid extractions

As mentioned before, extraction of samples employing organic solvents, although it requires higher amounts of solvents, is still used in nowadays sample preparation. In this Thesis liquid extraction has been employed in Chapter 1 to extract polar compounds from lyophilised samples employing a mixture of MeOH: H_2O. The suitability of polar mixtures of methanol-water has been broadly reported for the extraction and metabolomic analysis of polar compounds in plants [43, 45, 145] or polyphenols in fruit samples [20, 146]. In addition, in Chapter 3.2.2., EtAcO was employed to extract 5-HMF and patulin from fruit juices as its efficacy has been widely reported in several publications [147-150]. Also in Chapter 3.2.1, liquid-liquid extraction employing a mixture of MeOH: H_2O was used to extract polyphenols from freeze-dried fruit samples.

1.2.1.2. Microextraction techniques

The trend in nowadays analytical chemistry is to reduce and, when possible, to eliminate the use of solvents in the sample preparation step, which is together with analytical separation (especially in HPLC) the most solvent consuming steps in the

analytical process. For this purpose, from 1990's, several microextraction techniques have emerged. These techniques can be distinguished in two main groups depending on the nature of the extraction phase (solid or liquid).

a) Solid Phase Microextraction Techniques.

Solid-phase microextraction (SPME), in-tube solid-phase microextraction (IT-SPME), and stir bar sorptive extraction (SBSE) can be considered as the three main groups of microextraction techniques employing a solid as extraction phase (Figure 7). Within the scope of this Thesis two of these techniques, i.e. SPME and SBSE, have been used as will be further developed in Chapter 3. In regards of SPME, this technique was introduced in 1990 by Arthur and Pawliszyn [151]. SPME employs a fused silica fibre with an outer diameter of typically 150 μm which is coated with an (ad)sorbent layer up to 100 μm. This fibre can be inserted into the gaseous or liquid sample to carry out analysis of the Headspace (HS) or by direct immersion (DI), respectively. After exposure for a certain period of time of the fibre in the sample until the equilibrium is reached, the fibre is retracted and the SPME needle is desorbed in the hot inlet of the GC to carry out sample analysis. It is noticeable that currently exist several commercial coatings for SPME, which can be classified into four categories: by the type of coating, by the thickness, by polarity and by whether the coating is an adsorbent or an absorbent [152]. The coatings that are commercially available are polydimethylsiloxane (PDMS), polyacrylate (PA), carboxen (CAR), carbowax (CW), and divinylbenzene (DVB) in different combinations. Furthermore, in recent years, ionic liquids (IL) have appeared as alternative tuneable coatings due to this kind of compounds possess wide liquid ranges, low volatilities (negligible vapour pressure), good thermal stabilities, electrolytic conductivity, wide range of viscosities, adjustable miscibility, reusability, nonflammability and so on. One important feature of ILs is that varying the cation or anion may significantly affect physical and chemical properties [153-157].

Figure 7: Main solid-phase microextraction techniques [158].

SBSE was introduced in 1999 by Baltussen *et al.* [159]. The SBSE analytical devices are constituted by magnetic stir bars incorporated into a glass jacket typically coated with 24-126 µL of PDMS, a nonpolar polymeric phase characterised to promote hydrophobic interactions with target molecules, where the retention mechanism occurs mainly through Van-der-Waals forces but also hydrogen bonds can be formed with oxygen atoms of PDMS depending on the molecular structure of the analytes [160]. The main advantage of SBSE over SPME is that the volume of PDMS involved, in relation to the SPME fibres (up to 0.5 µL for 100 µm film thickness), promotes a lower phase ratio between the sorbent phase and the sample media, which provides an increasing capacity. SBSE allows also quantitative recoveries of the anlytes, especially for nonpolar solutes. Furthermore, this polymer is well-known to have thermo-stable properties, and a high degree of inertness. Moreover, its degradation products are very well known and can be easily identified with the MS. For these reasons, initial applications dealt with thermal desorption (TD) following by GC analysis [159]. On the contrary, this phase presents low enrichments for more polar compounds [161]. The fact that TD is performed at high temperatures in the TD unit reduces the availability of commercial coatings and only an ethylene glycol-silicone (EG-silicone) is currently supplied by Gerstel although lack of effective applications have been proposed [20]. An alternative to improve recoveries of polar compounds with the PDMS coating is to introduce a derivatization step prior to the extraction [162-164]. This approach improves the nonpolar characteristics of the more hydrophilic solutes and therefore, the affinity towards the polymeric phase, as well as the volatility for a better GC. Another variation to increase extractability of polar compounds using SBSE is the use of a multimode assay either in dual or sequential mode [165, 166]. As mentioned, SBSE was initially conceived to carry out the analysis of aqueous samples

in immersion mode [159]. However, a few years later, applications in HS mode were also introduced [167]. On the other hand, in the field of HPLC several coatings have emerged due to desorption of the analytes is performed in milder conditions by solvent back-extraction, which allows a higher number of sorptive phases to be employed due to the lower stability requirements [160, 161].

b) Liquid Phase Microextraction Techniques (LPME).

In LPME, the extraction is carried out between several microlitres of a water-immiscible solvent (extracting or acceptor phase) and several millilitres of an aqueous phase (donor phase) that contains the analytes of interest. As a consequence, important preconcentration factors and, as a result, low limits of detection (LODs) can be obtained. LPME comprises a good number of techniques as can be seen in Figure 8. These techniques can be divided into three main categories: single-drop microextraction (SDME) [168, 169] with main applications related to the analysis of essential oils components [170] and 3 fat-soluble vitamins in juices [171]; hollow-fibre LPME (HF-LPME) [172] with applications focused on the analysis of polyphenols in plant material [173, 174]; and dispersive liquid-liquid microextraction (DLLME) [175] with most of the existing research concerning endogenous compounds devoted to the analysis of essential oil components [176-178] and tea [179]. The lack of published papers in this field shows that there is still a broad field of knowledge to be run. In addition, each of these categories has several variations. Indeed, LPME, coupled with various instrumental analytical methods, has been increasingly and widely used in recent years [180].

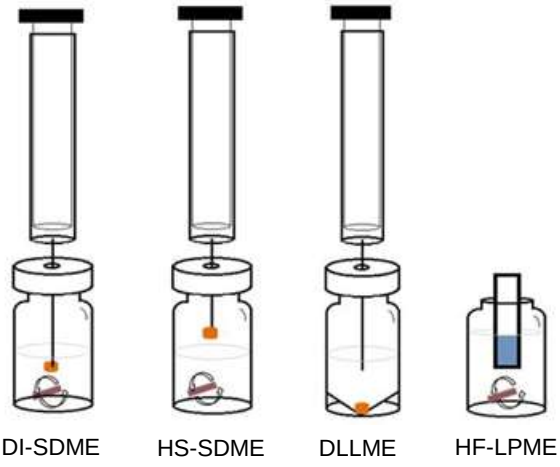

Figure 8: Main liquid-phase microextraction techniques [158].

SDME is based on the distribution of analytes between a microdrop of extraction solvent (usually few microliters) at the tip of a microsyringe needle and an aqueous sample phase containing the analytes. After extraction, the microdrop is retracted back into the microsyringe and injected into a chromatographic or electrophoretic system for further analysis [180]. Due to the instability of the drop when stirring is applied, this technique is recommended for HS sampling. For this reason, most of their applications in natural products analysis are related to this [181-186].

HF-LPME was developed with the aim to enhance single drop stability. The technique is based on a first analyte extraction into a supported liquid membrane (SLM) sustained in the pores of a hydrophobic porous hollow fibre (HF), which is generally made of polypropylene, and finally into an acceptor phase located inside the lumen of the HF, which was attached in both ends to respective needles of microsyringes. HF-LPME is probably the LPME most widely applied in natural products analysis, especially coupled to HPLC, due to the method performance is easier than that of SD-LPME in which drop stability is a crucial step and offers higher precision than DLLME, which involves higher contamination by sample impurities produced by the direct contact of the extraction solvent with the matrix [180].

DLLME was first introduced by Assadi *et al.* in 2006 [175] and it is based on a ternary component system, including disperser solvent, extraction solvent and aqueous phase sample containing the compounds of interest. The technique includes the following steps: (1) Injection of an appropriate mixture of extracting and disperser solvents into the aqueous sample, which contains the analytes. In this step, the extracting solvent is dispersed into the aqueous sample as very fine droplets and the analytes are enriched into it. Due to the large surface area between the extracting solvent and the aqueous sample, equilibrium is achieved quickly. Thus, the extraction is considered to be independent of time, being the most important advantage of this microextraction technique. (2) Centrifugation of the cloudy solution resulting in phase separation between the aqueous phase (top) and the chlorinated-organic phase containing the analytes (bottom). (3) analytes in the lower phase are determined by analytical instruments [187]. Since high dense water-immiscible solvents are used in DLLME, GC is the ideal technique to couple with this microextraction technique allowing direct injection of the extract into the GC injection port. As will be further developed in Chapter 3 of this Thesis, DLLME coupled with injection-port derivatization is a suitable technique to analyse the lipophilic fraction of fruit juice samples.

1.2.2. Derivatization methodologies

In general, derivatization reaction in GC can be classified taking into account two considerations: type of reaction and derivatization technique. In regards of the type of reaction, silylation, alkylation, and acylation are the processes most commonly employed. Silylation reagents consist of a trialkylsilyl, usually trimethylsilyl (TMS), that react with a

wide range of oxyanions to form thermally stable derivatives [38]. Silyl derivatives are probably the most widely derivatization reagents employed in GC due to their superior performance in terms of: 1) greater volatility and better thermostability; 2) most of silylation reaction are very fast; 3) silyl derivatives possesses great potential in conferring enhanced detectability, especially for MS detector [188]. It is important to notice that the reactivity of possible target groups for the silylation reagents decreases in the following order: alcohols >phenols >carboxylic acid >amine >amide [189]. In addition, primary alcohols and amines offer higher reactivity than secondary and tertiary alcohols and amines. Moreover, it is worth saying that the reactivity to hydroxyl groups varies for each reagent as following [38]:

TMSIm> BSTFA> BSA> MSTFA> TMSDEA> MSTA> TMCS (with base catalyst)> HMDS.

Alkylation refers to the replacement of the hydrogen of the polar groups in the analytes, e.g. -OH, -SH, -NH-, -COOH, -CONH-, with alkyl groups to yield more volatile ethers, thioethers, *N*-alkyl amines, esters, and *N*-alkyl amides, respectively. The principal alkylation reagents include diazomethane, tetraalkylammonium (TAA) salts, alcohols (acid as catalyst), and alkyl halides. Among these, TAA salts have acquired more popularity due to their high reaction rate, high derivatization efficiency and operation convenience [190, 191].

Acylation is usually employed to prepare acylated derivatives for -NH-, -OH and -SH groups using carboxylic acid, acid anhydride, acyl halide, and activated acyl amide as derivatization reagents. Acylation with acid anhydride requires the use of anhydrous conditions to avoid hydrolysis of the reagent. This reaction is rarely used in online derivatization because it requires longer reaction times and online processes require to be carried out very fast.

Considering the derivatization technique, conventional derivatization is carried out off-line in a glass vial of an appropriate volume by heating the mixture at an optimum temperature for a certain period of time, depending on the sample and compounds to analyse [38]. In recent years, online approaches have appeared in several variations, such as in-syringe derivatization [192, 193], in-port derivatization (Figure 9) [194-196], on-spot derivatization [197], solid-phase microextraction with on-fibre derivatization [198-200], liquid-phase microextraction with in situ derivatization [201, 202], and sorptive extraction with in situ derivatization [203-206]. It is noticeable that online approaches represent a reduction of solvents and reagents. In addition, these techniques usually tend to a higher degree of automation, which is generally traduced to an improvement of the repeatability and an important saving of time for sample preparation.

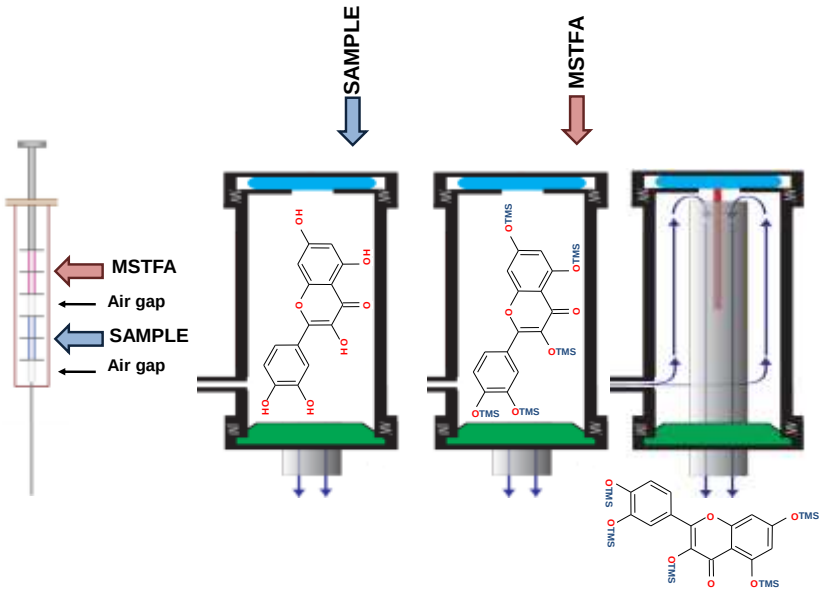

Figure 9: Schematic of an injection-port derivatization system.

1.2.3. Chromatographic techniques

Although some of the compounds analysed in this Thesis can be analysed employing HPLC, namely the group formed by high boiling compounds, GC-based techniques can also be used. Hence, the employed techniques can be divided between mono- and multimensional gas chromatography.

1.2.3.1. Monodimensional GC

Monodimensional GG is the typical configuration of a gas chromatograph in which a single capillary column is used. Through an interphase the column is connected to the detector such as flame ionization detector (FID) or thermal conductivity detector (TCD) to obtain chromatographic peaks. However, when more complex samples are analysed MS gives mass spectra, which are characteristic for each compound. Consequently, in addition to quantification, MS is very helpful for identification purposes. In mass spectrometers hyphened to a GC the ionization source most common is electron ionization (EI), in which energetic electrons interact with gas phase atoms or molecules to produce ions previous to their analysis in the detector. In the monodimensional

chromatography analyses carried out within the scope of this Thesis we used MS with quadrupole detector.

Current quadrupole detectors can be distinguished between single quadrupole (Q) and triple quadrupole in tandem (QqQ). Single quadrupoles can be operated in two different modes:

- Scan mode: performs a scan between a predefined mass range.
- Selected ion monitoring (SIM): in this mode only the selected ions for the target compounds are monitored. As a lower number of ions are scanned, more scan time is used for each ion resulting in better selectivity and lower limits of detection (LOD).

As shown in Figure 10, when using a QqQ instrument in the first quadrupole (Q1) the parent ions are selected on the basis of high abundancy and, if possible, high *m/z*. Secondly, in the collision cell (q2) parent ions are fragmented selecting the appropriate collision energy for each parent ion. Thirdly, in Q3 the daughter ions are selected from the fragments obtained on the basis of high abundancy and high *m/z* to achieve the lowest LOD. Finally, the selected transitions are checked in real samples to be test that they do not present any interference with other matrix compounds. Obviously, with QqQ better selectivity of the analytes is achieved resulting in lower LOD making this technique ideal for the quantitation of target compounds at low concentrations in complex extracts.

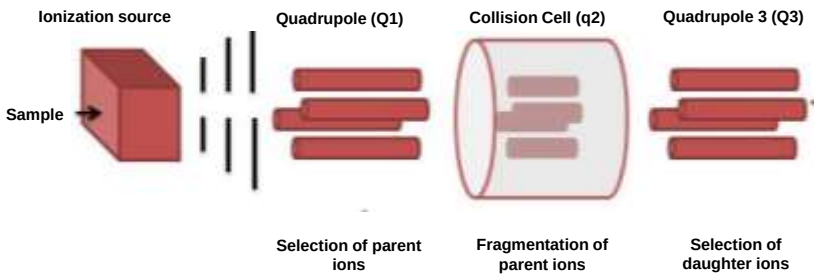

Figure 10: Schematic of a QqQ mass spectrometer.

1.2.3.2. Multidimensional GC

Multidimensional GC separations employ two or more gas chromatographic separations in a sequential manner. The separation produced by each dimension is maintained, at least partially, resulting in a resolving power higher than that of the individual stages [207]. Generally, multidimensional separations are divided into the following two categories:

a) Heart-Cutting two-dimensional gas chromatography (2D-GC).

Simmons *et al.* reported in 1958, by first time, 2D-GC [208]. This technique subjects a small portion of the sample to both separation columns. This is achieved employing a pressure switching device, called Dean's switch [209], which in its normal state does not allow the components of the first to enter in the second dimension. The interface is switched just prior to the elution of the fraction of interest, i.e. a fraction with coeluting components. Then, once the fraction is loaded in the secondary column it is switched back to the original state. Employing columns with different stationary phases, it is expected to resolve the coelutions deriving from the first dimension separation.

b) Comprehensive two-dimensional gas chromatography (GC×GC).

Liu and Phillips introduced GC×GC in 1991 [210]. Figure 11 shows a schematic of the GC×GC instrument used in this Thesis. Unlike 2D-GC, all the products are analysed by both columns. The two columns are connected with an interface called modulator, which allows an interval between transfers. This gap is known as modulation period. Modulators are still a matter of study and development and have evolved in the last years. However, most of commercial instrumentation uses a cryogenic modulator that employs liquid nitrogen as cryocooler. Another point to consider in GC×GC is the detector. As the peak widths obtained by GC×GC are narrower than in GC-MS, faster detectors are required. For this reason, most instruments employ time-of-flight mass spectrometry (TOFMS) because they are capable of producing full scans at rates greater than 100 Hz [207]. Regarding detectors, further research is also being done as new faster quadrupoles are being coupled to GC×GC achieving good scan performances [211, 212]. In addition, FID can also be used when quantification is required. Furthermore, Seeley *et al.* published an interesting review on the different instrument configurations employed regarding: the stationary phases used for column configuration, the detector, and the modulator and they concluded that the "standard" configuration is a GC×GC instrument fitted with a cryogenic modulator, a nonpolar × polar column configuration with a 50% phenyl-substituted secondary column, and a TOFMS detector [207]. As a consequence of the increased resolving power, this technique has been widely used in complex samples such as fuels and petrochemicals composition; environmental analysis (atmospheric, water, soils and sediments); foods, flavours, and fragrances; and biological studies [207].

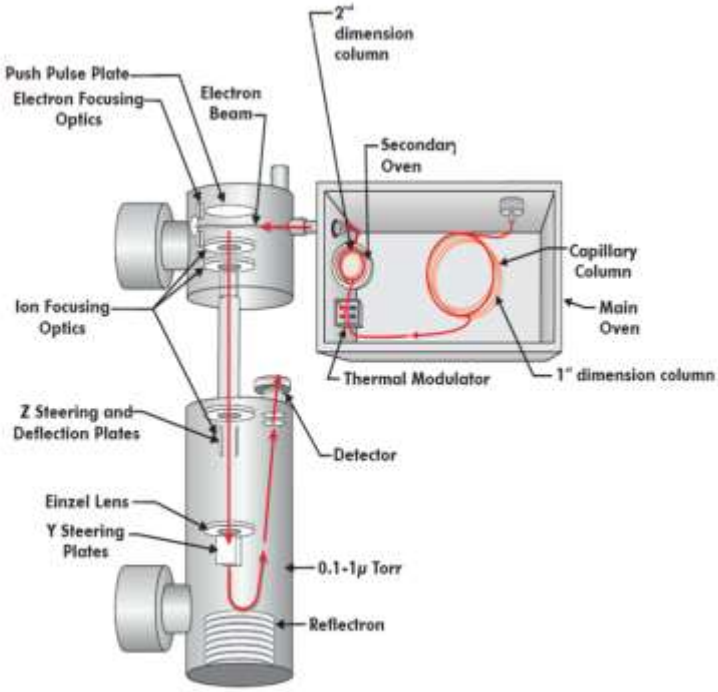

Figure 11: Schematic of the GC×GC instrument used in this Thesis (Adapted from Leco Corp. [213]).

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CHAPTER 2. OBJECTIVES AND ORGANIZATION

The main objective of this Thesis is to develop advanced analytical techniques based on gas chromatography coupled to mass spectrometry to analyse several classes of metabolomic compounds such as disaccharides, polyphenols, HMF and patulin, lipophilic compounds, and volatile and semi-volatile compounds in fruit and fruit-derived samples.

To achieve this objective several state-of-the-art techniques will be applied on each case depending on the analytical issue to deal with.

The experimental part of this thesis is organized as follows:

- Chapter 3.1.: consists of a comprehensive method based on two–dimensional gas chromatography combined with time–of–flight detection (GC×GC–ToF–MS) to analyse the disaccharide composition of apple and peach. The results of this Chapter have been published in *Phytochemical Analysis,* 2015, **26**, 279–286.

- Chapter 3.2.: includes three research papers which detail the analysis of polyphenols in fruits and juices, HMF and patulin, and the lipophilic fraction of fruit juices. The nexus between the three research papers is the use of injection-port derivatization to convert the compounds into trimethylsilyl derivatives. The results from Chapter 3.2. have been published in *Food Chemistry,* 2016, **204,** 210–217 and *Journal of Chromatography A,* 2016, **1453,** 99–104 and submitted to *Journal of Chromatography A.*

- Chapter 3.3.: is focused on the analysis of volatile and semi-volatile compounds in fruit fibres and peach juices, respectively. For this ppurpose, HS-SPME was used for the analysis of the volatile composition and a methodology employing SBSE was optimized for the direct immersion analysis of peach juice samples. The results from Chapter 3.3. have been submitted to *Journal of the Science of Food and Agriculture* and *Journal of Agricultural and Food Chemistry.*

CHAPTER 3. EXPERIMENTAL, RESULTS AND DISCUSSION

3.1. Comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry to assess complex samples

This Chapter is focused on the determination of the disaccharide composition in apple (*Malus domestica*) and peach (*Prunus persica*) varieties. Extraction of polar compounds was carried out employing an aqueous: methanol solution followed by evaporation of the extract under vacuum and derivatization by methoximation and trimethylsilylation [1, 2]. Considering the chromatographic part of the analysis, besides using a conventional monodimentional GC-MS method, bidimensional gas chromatography (GC×GC-ToF-MS) has been employed as was previously done in honey samples [3]. As it is well known, in addition to enhancing separation power, GC×GC allows lowering limits of detection due to the cryofocusing produced in the modulator [4]. As will be further discussed in the Chapter, only sucrose, the main disaccharide with a concentration about 100-fold greater than other disaccharides in the samples was detected if only employing a one-dimensional system.

Employing this technology several column configurations has been assessed including the "classic" non-polar/semi-polar, polar/non-polar and semi-polar/non-polar being the latter the one giving a better performance. With the use of the optimum configuration, ten disaccharides were identified including the abiotic stress biomarker α,α-trehalose [5], based on analytical standards, retention indexes and mass spectra. In addition, the application of the methodology to fruit samples revealed that apple and peach present significant differences in disaccharide composition, while when comparing the composition among varieties this was found very similar.

The results of this Chapter have been published in *Phytochemical Analysis* 2015, **26**, 279-286.

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3.1.1. Comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry to assess the presence of α,α-trehalose and other disaccharides in apple and peach

COMPREHENSIVE TWO-DIMENSIONAL GAS CHROMATOGRAPHY TIME-OF-FLIGHT MASS SPECTROMETRY TO ASSESS THE PRESENCE OF α,α-TREHALOSE AND OTHER DISACCHARIDES IN APPLE AND PEACH

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Abstract

Carbohydrates are important constituents in fruits. Among the carbohydrates, disaccharides have rarely been studied in apple and peach. Indeed, the abiotic stress biomarker and preservation agent α,α-trehalose is a disaccharide. This work aims to establish a comprehensive method based on two-dimensional gas chromatography combined with time-of-flight detection (GC×GC-ToF-MS) to analyse the disaccharide composition of apple and peach. Sample preparation was based on aqueous-methanolic extraction of the analytes, followed by oxime formation and trimethylsilylation of the disaccharides. First, three columns were tested with standards on the 1-dimensional system. Next, to perform the sample analysis using GC×GC-MS (which offers significant advantages over conventional GC because it allows higher separation efficiencies), various column configurations were assessed on the two-dimensional system to obtain enhanced separation and low detection limits. The column sets tested included non-polar/semi-polar, semi-polar/non-polar and polar/non-polar. Using the method that proved to be more efficient, namely the method developed with the semi-polar/non-polar configuration, ten disaccharides were identified, based on analytical standards, retention index and mass spectra. These compounds were quantified in several varieties of apple and peach fruit using the developed GC×GC method and linear curve calibration, resulting in substantial differences among the fruits. However, cultivars within the fruits exhibited no significant differences.

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***Keywords**: Comprehensive two-dimensional gas chromatography (GC×GC), α,α-trehalose, disaccharides, fruit.*

1. Introduction

Fruit contains high amounts of carbohydrates; for example, these molecules account for approximately 15% of the dry matter content of apple (Füzfai *et al.,* 2004), while in peaches, they represent 63-68% of the dry matter at harvest (Pavel *et al.,* 1993). Carbohydrates include compounds such as monosaccharides, sugar alcohols, sugar acids, and disaccharides. Among these compounds, disaccharides are a challenge for analysts because of the similarity of structures and the presence of some of these compounds at low concentrations in fruit matrices (Sanz *et al.,* 2002). This similarity is a consequence of the fact that many disaccharides are commonly only differentiated by the position and configuration of the glycosidic linkage. The importance of disaccharides in fruit is especially noticeable in the case of sucrose, which is the most abundant sweetener in fruits, and in the case of α,α-trehalose, which is considered to be an abiotic stress biomarker (Iordachescu *et al.,* 2008; Lee *et al.,* 2003) and a preservation agent (Albanese *et al.,* 2007; Ohtake *et al.,* 2011). Moreover, short-chain carbohydrates vary in their digestibility and subsequent absorption. Those short-chain carbohydrates that are poorly absorbed exert osmotic effects in the intestinal lumen, thus increasing its water volume, and are rapidly fermented by bacteria with the consequent gas production (Shepherd *et al.,* 2013). These findings increase the need for powerful analytical techniques to determine the presence of these compounds in foodstuff with high accuracy (Jovanovic-Malinovska *et al.,* 2014).

HPLC is the most widely used technique to analyse carbohydrates in fruit. However, gas chromatography-mass spectrometry (GC-MS) exhibits acceptable performance, and MS data can be useful for identification purposes when commercial analytical standards are not available. Nevertheless, GC requires previous derivatisation of the samples to increase the volatility of carbohydrates. This step is usually achieved by oximation of the free carbonyl group of reducing disaccharides, thus suppressing the anomeric centre, and only two derivatives, *syn* (*E*) and *anti* (*Z*), are formed. This step is then followed by trimethylsilylation, although alkylation is also performed in some cases (Blakeney *et al.,* 1983). The analysis of derivatised compounds is commonly performed using non-polar capillary columns with poly(dimethyl-phenyl siloxane) coatings (Molnár-Perl 2000). Novel columns coated with ionic liquids (ILs), which exhibit unique physicochemical properties, have recently been developed to be used in analytical chemistry. ILs, in addition to their physiochemical properties, also provide multiple solvation interactions with molecules (Armstrong *et al.,* 1999).

In addition, comprehensive two-dimensional gas chromatography-mass spectrometry (GC×GC-MS) offers significant advantages over conventional GC because it allows for higher separation efficiencies. Furthermore, cryofocusing caused by the modulator leads to higher detectability of less abundant compounds (Koek *et al.,* 2008). These two advantages make this technique a valuable tool for the analysis of structurally similar compounds, especially those present at low concentrations. GC×GC-MS has been applied to several complex mixtures (Mondello *et al.,* 2008) and to food analysis (Herrero *et al.,* 2009). Nevertheless, to the best of our knowledge, only one study has described the

application of a GC×GC procedure to separate disaccharides in a foodstuff, namely honey. However, no quantisation was performed (Brokl *et al.,* 2010).

Here, we sought to assess the performance of GC×GC-MS to determine the disaccharide composition of fruit, specifically of apple (*Malus domestica*) and peach (*Prunus persica*), one of the most prominent fruit crops in mid-latitude climatic zones and one of the most widely produced stone fruits. Three capillary columns were used to first separate the analytical standards and then to separate the fruit samples. The column performance was evaluated using typical quality parameters. The columns included the common non-polar column coated with a poly (95% dimethyl- 5% phenyl siloxane) and a semi-polar column coated with poly (50% dimethyl- 50% phenyl siloxane). In addition, a recently launched more polar column coated with an IL stationary phase 1,12-di(tripropylphosphonium)dodecane bis(trifluoromethylsulfonyl)imide, named SLB-IL60, was also tested as an alternative to conventional polyethylene glycol (PEG) columns, which provides a polarity similar to this IL phase.

In this study, the assessment of the disaccharide composition in several fruits was performed. To perform this assessment, GC×GC was the chromatographic technique used because it offers enhanced separation and lower limits of detection compared to conventional gas chromatography. Regarding the GC×GC technique, three column sets were tested for the two-dimensional system, and compounds were identified on the basis of the commercial standards, when available, or the retention index and mass spectrometry data.

2. Experimental section

2.1. Materials and reagents

All solvents were of analytical purity. *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) and methoxyamine hydrochloride (MEOX) were purchased from Sigma-Aldrich (Buchs, Switzerland).

Analytical standards of palatinose (6-*O*-α-D-glucopyranosyl-D-fructose), cellobiose (4-*O*-β-D-glucopyranosyl-D-glucose), maltose (4-*O*-α-D-glucopyranosyl-D-glucose), turanose (3-*O*-α-D-glucopyranosyl-D-fructose), melibiose (6-*O*-α-D-galactopyranosyl-D-glucose), gentiobiose (6-O-β-D-glucopyranosyl-D-glucose), maltitol (4-O- α-D-glucopyranosyl-D-glucitol), and isomaltose (6-*O*-α-D-glucopyranosyl-D-glucose) were purchased from TCI (Zwinjdrecht, Belgium). Sucrose (2-*O*-α-D-glucopyranosyl-β-D-fructofuranoside) and α,α-trehalose (1-*O*-α-D-glucopyranosyl-α-D-glucopyranoside) were purchased from Sigma-Aldrich. Maltulose (4-*O*-α-D-glucopyranosyl-D-fructose) was purchased from Alfa Aesar GmbH (Karlsruhe, Germany), and isomaltitol (6-O-α-D-glucopyranosyl-D-glucitol) was purchased from Carbosynth Ltd. (Compton, United

Kingdom). Stock solutions of solid product were prepared in a water-methanol (70: 30) solution.

2.2. Samples

The samples were three commercial apple cultivars, namely Granny Smith, Golden Delicious and Royal Gala, purchased from a local supermarket in Spain, and three commercial peach cultivars, namely Amarillo de Septiembre, Miraflores, and Andross, harvested in 2012 in various orchards in Spain and supplied by a fruit company.

Samples were ground and kept frozen at -80 ºC before being submitted to lyophilisation.

2.3. Extraction and derivatisation procedure

Aliquots of 50-100 mg of frozen tissue were placed in a 2.0-mL Eppendorf vial and freeze-dried for 24 h. A volume of 10 µL of a 2 mg/mL solution of isomaltose (IS) was added to each sample. Subsequently, 500 µL of a H_2O-methanol (70:30) solution was added to each vial. The mixture was vortexed for 20 min and then centrifuged at 20,000 × g for 5 min. A volume of 40 µL of the extract was placed into a chromatography vial containing a glass insert. The extract was dried for 30 min at room temperature using a SpeedVac (Thermo, Asheville, NC, USA) apparatus. The residue was then suspended in 30 µL of a solution of MEOX in pyridine (20 mg/mL). The vial was sealed with a cap, vortexed until the residue was completely solved, and then incubated in a ThermoMixer (Eppendorf AG, Hamburg, Germany) for 1 h at 40 ºC. Next, the vial was removed, and then 80 µL of MSTFA was added. The sample was vortexed and incubated at 40 ºC for another 1 h. Finally, a mixture of alkanes (C7-C40) (Sigma-Aldrich) was added to build a retention time index (RTI) method. Each sample extraction was performed in triplicate.

2.4. GC-MS analysis

Several disaccharide standards at a concentration of 500 µg/mL to cover a wide range of glycosidic moieties and linkages were used to test the following three systems: a non-polar column (30 m × 0.25 mm ID × 0.25 µm d_f) coated with poly (95% dimethyl-5% phenyl siloxane) named DB-5ms (Agilent Technologies, Santa Clara, CA, USA); a semi-polar column (30 m × 0.25 mm ID × 0.25 µm d_f) coated with poly (50% dimethyl-50% phenyl siloxane), named ZB-50 (Phenomenex Inc., Torrance, CA, USA); and an IL-based column (30 m × 0.25 mm ID × 0.20 µm d_f), referred to as SLB-IL60 (Supelco, Bellefonte,

PA, USA). The latter was the most polar, exhibiting a polarity similar to PEG columns, but with a unique elution pattern as a result of its different phase composition.

GC analyses were performed in a 6890 gas chromatograph equipped with an MSD 5973 mass detector, both from Agilent Technologies, using helium as the carrier gas.

A volume of 1 µL of analytical standards was injected in split mode (1:10) at an injector temperature of 280 ºC. The quadrupole mass spectrometer was operated in electron ionisation (EI) mode at 70 eV in scan mode (mass range from *m/z* 50 to 600). The source and transfer line were set at 250 ºC and 300 ºC, respectively. Data acquisition and processing were performed using Agilent MSD Chemstation software.

Finally, all of the columns were tested with the derivatised commercial standards to achieve its best performance. The optimised conditions for each column were as follows. For the DB-5ms column: 1.1 mL/min and an oven programme of 260 ºC isothermal (20 min). For the ZB-50 column: 1.1 mL/min flow; 100 ºC (1 min), 30 ºC/min to 230 ºC, and 4 ºC/min to 260 ºC (10 min). For the SLB-IL60 column: 1.2 mL/min flow; 150 ºC (5 min) and then a ramp of 10 ºC/min to 260 ºC (1 min).

Given the higher concentration of sucrose, this disaccharide was measured using the column DB-5ms with the previously mentioned oven conditions with a split ratio of 1:100 and a linear calibration curve constructed using a commercial standard of sucrose.

2.5. GC×GC-MS analysis

Table 1 presents the three sets of columns used to determine the disaccharide composition of the fruit samples. The method was optimised to prevent overlap of sucrose with minor disaccharides. In fact, sucrose is the main disaccharide in fruit, and it has the lowest retention time of all disaccharides. Thus, mass acquisition with the GC×GC-MS system started after the sucrose elution time.

Using a CombiPal autosampler (Gerstel, Mülheim-an-der-Ruhr, Germany), 1-µL aliquots of samples were injected into a GC×GC-ToF-MS system composed of a 6890 gas chromatograph (Agilent Technologies) and a Pegasus 4D ToF MS (LECO Corp., St. Joseph, MI, USA). The system was equipped with a secondary oven for independent temperature programming of the second-dimension column and a quad-jet dual-stage cryomodulator. Liquid nitrogen was used for cryofocusing the analytes eluting from the first dimension (1D) column, whereas resistively heated air was used for releasing and re-injecting these compounds into the second dimension (2D) column.

The injector temperature was set at 290 ºC, and 1-µL sample volumes were injected in splitless mode. Helium was used as a carrier gas at a flow rate of 1 mL/min.

The transfer line temperature was 250 ºC, and the source was set at 200 ºC. The detector voltage was 1600 V, and the EI voltage was 70 eV. The scan range was 250 spectra/s over a mass range of 50 to 600 a.m.u.

Table 1: Column sets used for sample analysis in the GCxGC system.

Configuration	Stationary phase	Dimensions (length (m) × i.d. (mm) × df (µm))
Non-polar/semi-polar	DB-5ms/Rtx17	(30 x 0.25 x 0.25)–(1.2 x 0.1 x 0.10)
Semi-polar/non-polar	ZB-50/DB-5ms	(30 x 0.25 x 0.25)–(1.2 x 0.1 x 0.18)
Polar/non-polar	SLB-IL60/DB-5ms	(30 x 0.25 x 0.20)–(1.2 x 0.1 x 0.18)

Data acquisition, instrument control, and peak quantisation were performed using the ChromaTof software package version 3.2 (LECO). Positive compound identification was considered when the retention index and the MS spectra were consistent with those of authentic standards analysed under the same experimental conditions. Compound identifications were considered to be tentative when only experimental MS spectra were available and the lack of standards did not allow for direct comparison. In this case, the GOLM Metabolome Database (Hummel *et al.,* 2010), which provides a high number of TMS-derivatised compounds acquired using a time-of-flight detector, was also used. For quantification purposes, *m/z* 73, the most intense mass fragment for derivatised compounds, was selected for each peak. Chromatographic peaks with an S/N >10 were considered for peak area integration. Quantisation of the disaccharide content was performed using a linear calibration curve constructed with commercially available standards. When standards were not available for a certain compound, the calibration curves of standards with a similar structure were used.

Semi-polar/non-polar column configuration, ZB-50/DB-5ms. The oven programme for this configuration was as follows: start at 220 ºC (held for 12 min), ramp up to 240 ºC at 2 ºC/min, and then ramp up to 300 ºC at 10 ºC/min (held for 7 min). The secondary oven followed the same programme, but with an offset of 20 ºC, and the modulator period was 4 s at a temperature offset of 50 ºC. The solvent delay was 11 min.

Non-polar/semi-polar column configuration, DB-5ms/Rtx17. Here, we used as a first column the previously described DB-5ms, and we used as a second column a semipolar column with 1.2 m × 0.1 mm ID × 0.10 µm d_f, coated with the stationary phase poly (50% dimethyl- 50% diphenylsiloxane), named RTX17, from Restek Corporation (Bellefonte, PA, USA).

The oven programme for this configuration was as follows: start at 100 ºC (held for 1 min), ramp up to 260 ºC at 30 ºC/min, and then ramp up to 300 ºC at 4 ºC/min (held

for 1 min). The secondary oven offset was 10 ºC, and the modulator period was 2 s at a temperature offset of 30 ºC. The solvent delay was 13 min.

Polar/non-polar column configuration, SLB-IL60/DB-5ms. The oven programme for this configuration was as follows: start at 100 ºC (held for 1 min), ramp up to 200 ºC at 20 ºC/min (held for 1 min), and then ramp up to 225 ºC at 4 ºC/min. The secondary oven offset was 22 ºC, and the modulator period was 5 s at a temperature offset of 67 ºC. The solvent delay was 7.5 min.

3. Results and discussion

3.1. GC-MS method development using commercial standards

Initially, commercial standards of disaccharides were injected into the GC-MS system to assess the performance of the DB-5ms, ZB-50 and SLB-IL60 columns in 1D separation. Quality parameters, such as the retention index, were calculated in the first dimension by linear interpolation of the retention times of the analytes within the alkanes standard mixture, following the method of van de Dool and Katz (van Den Dool *et al.,* 1963). The peak width and symmetry, as measured by the tailing factor from the peak width values at 5% peak height, were calculated for each of the standards in each column in the optimised separation conditions (Table 2). For the SLB-IL60 column, direct injection with reagent resulted in the appearance of artefacts in the chromatogram. To avoid these artefacts, evaporation and reconstitution of the sample with hexane prior to injection was mandatory. The three columns exhibited acceptable performance in separating the standards, although some co-elutions occurred, as previously reported (Sanz *et al.,* 2004) for polysiloxane-coated columns. In addition, the IL column provided the best separation between sugars with (1→6) linkage and the other disaccharides. This column was also the only one that completely separated α,α- trehalose. Regarding other quality parameters, the mean peak width was 0.44 min (0.20-0.71 min) in DB-5ms, 0.25 min (0.10-0.41 min) in ZB-50, and 0.19 min (0.11-0.27 min) in SLB-IL60, with the latter providing the optimum chromatographic peaks. The symmetry parameter revealed slight peak tailing for the ZB-50 and SLB-IL60 columns, with mean values of 1.39 (0.8-1.67) and 1.56 (1.12-1.94), respectively, which are also acceptable values for this parameter. This parameter was closer to that of DB-5ms, which exhibited a mean value of 1.19 (0.98-1.67).

Table 2: Analytical standard quality parameters determined by GC-MS (1D) for each of the columns.

Compound	Peak[a]	DB-5ms			ZB-50			SLB-IL60		
		RI	Width (min)	Symmetry	RI	Width (min)	Symmetry	RI	Width (min)	Symmetry
Sucrose		2603.3	0.38	1.04	2633.4	0.21	0.98	2604.8	0.27	1.12
Cellobiose	E	2671.3	0.31	0.96	2664.4	0.15	1.67	2717.5	0.16	1.38
	Z	2705.6	0.38	1.07	2695.2	0.14	1.35	2777.5	0.12	1.22
α, α -Trehalose		2712.6	0.36	1.08	2761.3	0.28	1.17	2698.8	0.15	1.42
Maltose	E	2714.7	0.47	1.09	2750.0	0.30	1.44	2710.0	0.14	1.51
	Z	2741.6	0.45	1.07	2775.0	0.24	1.24	2760.0	0.11	1.81
Turanose	1	2724.7	0.41	1.25	2770.2	0.29	1.63	2748.8	0.13	1.85
	2	2752.4	0.40	1.29	2798.4	0.29	1.35	2776.3	0.14	1.87
Maltulose	1	2701.7	0.20	1.32	2745.2	0.10	1.51	2713.8	0.15	1.41
	2	2710.0	0.43	1.08	2750.0	0.24	1.24			
Gentiobiose	E	2799.6	0.58	1.21	2840.0	0.22	1.43	2909.1	0.28	1.79
	Z	2822.4	0.38	1.60	2854.5	0.31	1.09	2946.3	0.28	1.45
Palatinose	1+2	2827.4	0.54	1.06	2859.3	0.41	1.58	2898.2	0.26	1.32
Melibiose	E	2846.8	0.71	1.12	2871.7	0.31	1.64	2944.5	0.27	1.94
	Z	2877.6	0.48	1.22	2891.0	0.19	1.38	2989.6	0.21	1.45
Isomaltose	E	2851.2	0.61	1.15	2880.7	0.31	1.65	2922.0	0.20	1.91
	Z	2884.6	0.39	1.58	2903.7	0.34	1.24	2970.7	0.22	1.53
Mean±SD			0.44 ± 0.12	1.19 ± 0.17		0.21 ± 0.08	1.39 ± 0.20		0.19 ± 0.06	1.56 ± 0.25

[a] Syn (*E*) or anti (*Z*) isomers were assigned according to the results of Funcke and von Sonntag (Funcke et al., 1979). When assignment was not possible, the isomers were named 1 and 2.

3.2. Assessment of the GC×GC performance in fruit samples

The application of GC×GC to analyse disaccharides in apple and peach samples is useful because this technique shows not only higher separation power but also improved limits of detection. Figure 1 shows for the same sample an expanded chromatogram using 1D and 2D chromatography where, using 2D, an improvement in separation and sensitivity was achieved, leading to the detection of additional minor content disaccharides.

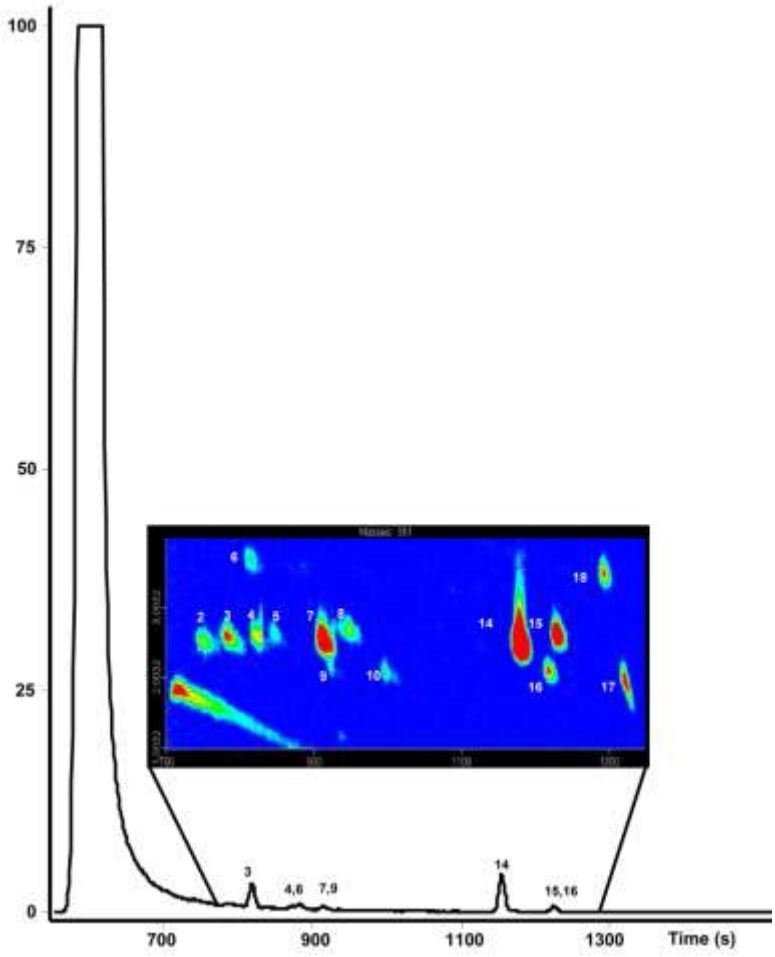

Figure 1: Overlay of 1D and 2D chromatograms (splitless mode) at *m/z* 361 of an apple sample. The peaks are numbered according to Table 3.

Table 1 presents the three column configurations used to analyse the samples on the basis of the results obtained in the 1D GC-MS system.

The DB-5ms/Rtx17 (non-polar/semi-polar) configuration provided very little increase in the separation efficiency (Figure 2a). The peaks were almost entirely distributed across the 1D space. Therefore, this configuration provided lower detection limits than 1D. In contrast, in the ZB-50/DB-5ms (semi-polar/non-polar) (Figure 2b) configuration, satisfactory separation of the peaks along the 2D space was achieved.

The SLB-IL60/DB-5ms (polar/non-polar) (Figure 2c) configuration also provided acceptable occupation of the 2D space. However, the peaks with a lower retention time were in close proximity to that of sucrose, which appeared at a concentration 100-fold higher. The fact that sucrose was present at a higher range of concentration produced overlapping peaks between sucrose and other disaccharides with low retention time. Non-polar/polar and semi-polar/non-polar configurations were previously reported to provide good performance for the separation of mixtures of derivatised polar compounds (Gao *et al.,* 2010). Moreover, the present study is the first to test an IL-coated column for the analysis of polar TMS-derivatised compounds. Using the IL column for the first dimension, an increase in system orthogonality was expected due to the use of a completely different phase that provides dissimilar separation mechanisms.

Finally, to prevent the overlapping with sucrose mentioned above, we selected the configuration consisting of ZB-50 in the first dimension and DB-5ms in the second for further analysis of samples.

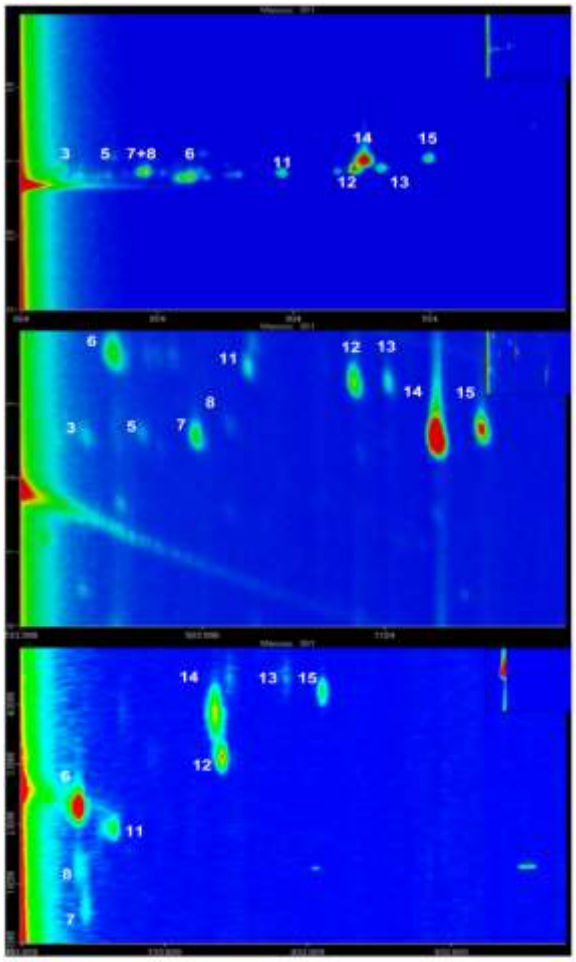

Figure 2: GC×GC contour plot of a peach sample (Amarillo de Septiembre) at *m/z* 361 with various column sets: a) DB-5ms/Rtx17; b) ZB-50/DB-5ms; c) SLB-IL60/DB-5ms. The peaks are numbered according to Table 3.

Table 3: Fragmentation patterns of the trimethylsilyl-oxime derivatives of the disaccharides found in the fruit samples.

Peak	Compounds	RI±SD	Fragment : *m/z*												
			147	**191**	**204**	**217**	**243**	**271**	**305**	**307**	**319**	**345**	**361**	**437**	**480**
2	(2,1)-β-Fructofuranosyl-fructofuranose[b]	2689.0 ± 2.0	42	4	15	100	0	1	0	7	1	0	7	2	2
3		2710.6 ± 2.2	53	4	0	100	2	0	0	8	2	0	11	2	2
4	Fructofuranosyl-fructofuranose-1[c]	2728.3 ± 1.4	54	5	2	100	2	2	0	0	0	0	21	1	0
5	α,α-Trehalose[a]	2745.9 ± 1.3	94	100	33	69	18	21	3	1	7	1	89	0	0
6	Cellobitol[b]	2726.7 ± 2.8	83	17	100	50	3	0	0	0	2	10	13	0	0
7	Fructofuranosyl-fructofuranose-2[c]	2774.1 ± 1.3	51	5	0	100	4	4	0	2	1	0	17	4	4
8		2793.5 ± 1.9	43	8	4	100	2	3	0	0	0	0	19	3	4
9	Fructofuranosyl-fructofuranose-3[c]	2779.4 ± 1.5	63	45	90	100	2	11	4	0	0	0	14	5	2
10		2816.4 ± 1.0	54	28	72	100	3	6	0	0	0	0	11	5	2
11	Maltitol[a]	2804.3 ± 1.0	89	19	100	47	3	1	0	1	7	5	12	0	0
12	Pyranosyl-pyranose-1[c]	2863.3 ± 1.8	88	20	100	45	13	13	3	2	6	0	40	0	2
13		2883.3 ± 2.1	76	20	100	49	10	10	3	2	7	0	35	0	2
14	Isomaltose (IS)	2909.2 ± 1.4	100	23	98	47	12	12	2	5	1	0	70	0	2
15		2933.7 ± 2.2	100	24	95	42	13	13	3	3	4	0	54	0	1
16	Pyranosyl-pyranose-2[c]	2926.7 ± 1.4	41	30	100	24	4	2	1	0	1	0	7	0	0
17		2979.4 ± 1.0	36	29	100	16	3	2	1	0	0	0	7	0	0
18	Pyranosyl-pyranosyl alcohol[c]	2966.6 ± 0.9	56	29	100	36	3	2	6	0	1	4	3	0	0

IS: Internal standard.

[a] Compounds identified by co-elution with chemical standards.

[b] Compounds tentatively identified by mass spectra and retention index coincidence with the GOLM database.

[c] Compounds tentatively assigned on the basis of their mass spectra.

3.3. Sample compound identification

Initially, a peach sample (Amarillo de Septiembre) and an apple sample (Golden Delicious) were studied to identify the disaccharides present in each fruit variety. Figure 3 shows the disaccharide composition of peach (2a) and apple (2b) at *m/z* 361 (Molnár-Perl *et al.,* 1997). This *m/z* value is characteristic of silylated disaccharides corresponding to a sugar ring with a loss of $OSi(CH_3)_3$, (*m/z*: 451-90=361). Apart from the internal standard isomaltose (peaks 14 and 15), positive compound identification was feasible only for the following peaks: peak 5, corresponding to α,α-trehalose, and peak 11, corresponding to maltitol. Peaks 2 and 3 presented almost identical mass spectra and high similarity to (2,1)-β-fructofuranosyl-fructofuranose from the library. The presence of fragment *m/z* 480 is indicative of a reducing disaccharide furnished by the moieties of an open chain structure (*m/z* 553-73=480). Peak 4 was also similar to peaks 2 and 3 but with the absence of *m/z* 307 and *m/z* 319. The presence of a single peak and fragment *m/z* 437 are indicative of a non-reducing disaccharide containing a fructose unit (Füzfai *et al.,* 2008). Peak 6, which appeared in both fruit cultivars, was tentatively assigned to cellobitol by comparing mass spectra and retention indices with the GOLM database. The presence of fragment *m/z* 345 is indicative of a disaccharide alcohol, as reported (Füzfai *et al.,* 2008) for sugar alcohols. Peaks 7 and 8 had the same mass spectra and were found in both fruit cultivars. Consequently, these peaks were assigned as the two TMS-oxime isomers derived from a reducing disaccharide formed by two fructofuranosyl rings. Peaks 9 and 10 were found only in the apple samples and were assigned to a disaccharide composed by two fructofuranosyl rings. Peaks 12 and 13 were only found in peach samples and correspond to a pyranosyl-pyranose disaccharide. Peaks 16, 17 and 18 were detected only in apple samples, with peaks 16 and 17 being the two TMS-oximes of a pyranosyl-pyranose disaccharide, while peak 18 was probably a disaccharide alcohol formed by two pyranoside units as a result of the absence of *m/z* 437 and the presence of *m/z* 345.

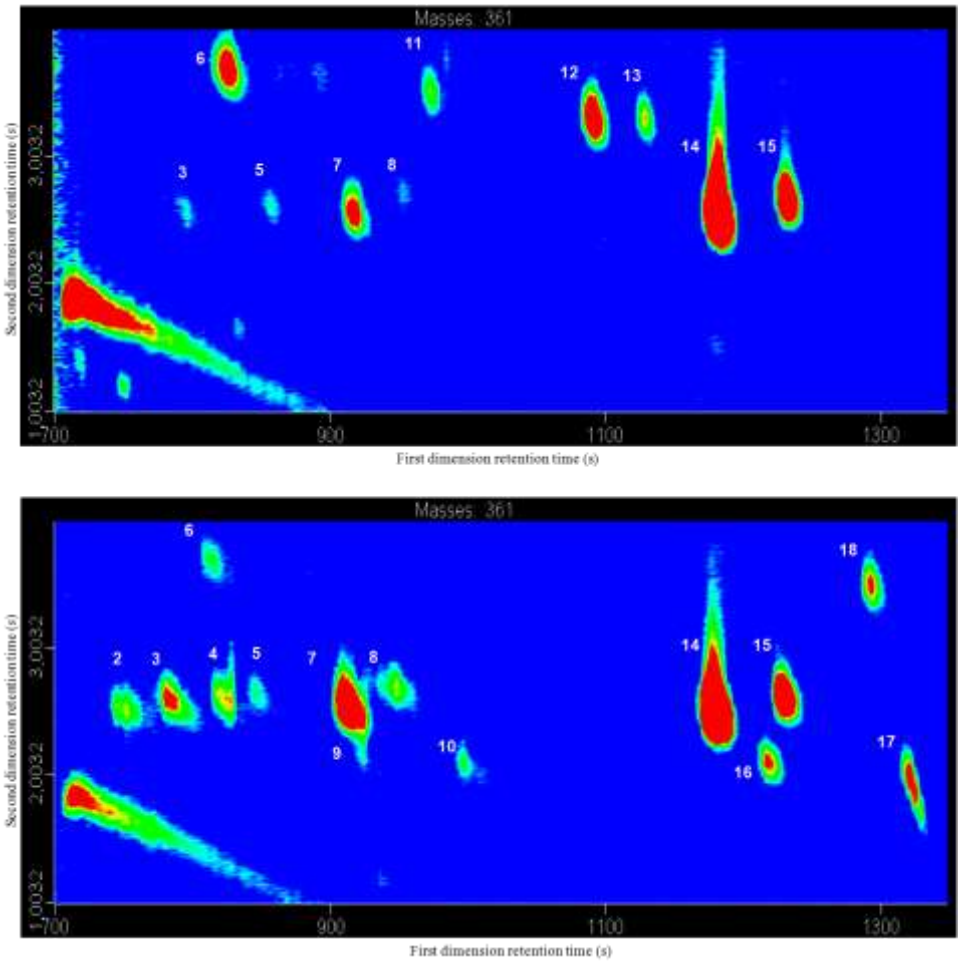

Figure 3: GC×GC contour plot (*m/z* 361) using the optimised method with ZB-50/DB-5ms of: a) peach sample (Amarillo de Septiembre); and b) apple sample (Golden Delicious). The peaks are numbered according to Table 3.

3.4. Quantification of the sample compounds

Finally, the method was applied to the quantitative analysis of three apple cultivars, namely Golden Delicious, Granny Smith, and Royal Gala, and three peach cultivars, namely Amarillo de Septiembre, Miraflores and Andross. Table 4 presents the calibration curve data over the concentration range of each of the standards used, as well as the LOD calculated at the concentration where S/N=3. Linear behaviour over the

concentration range studied for each of the standards varied between r^2=0.968 in isomaltose and r^2=1.000 in maltitol, and these values are acceptable for a TOF-MS detector, which usually has a lower dynamic range compared to a quadrupole detector (Santos *et al.,* 2003). The LOD for the standards used for the calibration curves ranged from 27 to 180 ng injected into the column. Lower LOD values were obtained for the compounds with a single chromatographic peak, namely non-reducing disaccharides (α,α-trehalose) and disaccharide alcohols (maltitol and isomaltitol), while the LOD values obtained for reducing disaccharides were slightly higher.

Table 4: Calibration curve data of commercially available standards used for quantification.

Compound	Molecular formula	Concentration range (µg/mL)	Equation	r^2	LOD[a]
α,α- Trehalose	$C_{12}H_{22}O_{11}$	11.5-0.2	y=298.9x + 19.8	0.995	27
Turanose	$C_{12}H_{22}O_{11}$	23.1-0.4	y=285.8x - 279.9	0.985	69
Maltitol	$C_{12}H_{24}O_{11}$	11.0-0.2	y=294.7x - 19.9	1.000	51
Melibiose	$C_{12}H_{22}O_{11}$	22.6-0.4	y=273.6x - 106.1	0.978	160
Maltose	$C_{12}H_{22}O_{11}$	6.8-0.4	y=162.3x + 43.4	0.981	180
Gentiobiose	$C_{12}H_{22}O_{11}$	11.9-0.4	y=293.0x - 31.4	0.983	170
Isomaltitol	$C_{12}H_{24}O_{11}$	6.9-0.2	y=191.6x - 82.7	0.990	86
Isomaltose	$C_{12}H_{22}O_{11}$	11.8-0.4	y=177.1x - 86.9	0.968	170

[a] Expressed as ng injected in column and calculated as S/N=3

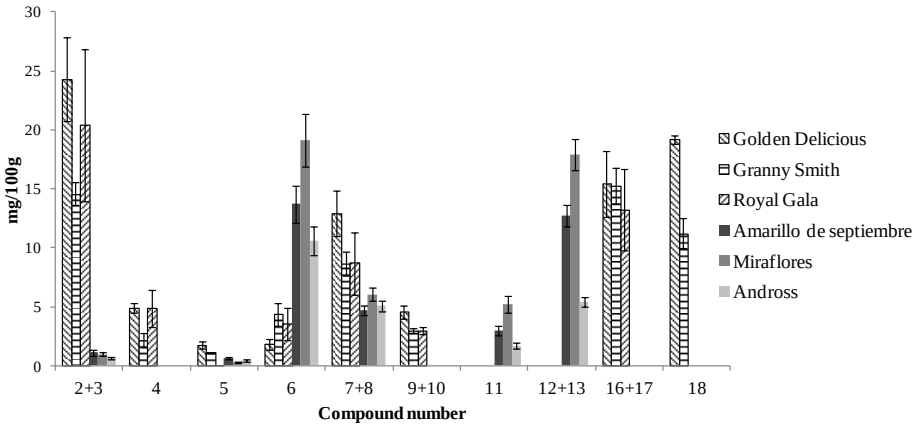

Figure 4: Disaccharide composition of apple and peach cultivars. Sucrose is not shown because of its higher concentration range. The peaks are numbered according to Table 3.

When the commercial standards were not available, we used the calibration curves for similar compounds with a close retention time and consequently similar structure. In this regard, the calibration curve of maltose was used for the quantisation of (2,1)-β-fructofuranosyl-fructofuranose, α,α-trehalose for fructofuranosyl-fructofuranose-1, turanose for fructofuranosyl-fructofuranose-2 and fructofuranosyl-fructofuranose-3, maltitol for cellobitol, melibiose for pyranosyl-pyranose-1 and pyranosyl-pyranose-2, and isomaltitol for pyranosyl-pyranosyl alcohol.

The sucrose content was measured using a GC-MS system and was found to have dry weight contents of 14.86, 7.38, and 33.07 mg/g in Golden Delicious, Granny Smith, and Royal Gala apple cultivars, respectively. Regarding peach cultivars, contents of 46.44, 15.55 and 12.20 mg/g were found for Amarillo de Septiembre, Miraflores and Andross, respectively.

With respect to the content of minor disaccharides (Figure 4), of note, this is the first study to detect α,α-trehalose in apple and at amounts slightly higher than those in peach, a matrix in which it has previously been described (Lombardo *et al.,* 2011). This study also reported new minor disaccharides and disaccharide alcohols in apple and peach samples.

(2, 1)-β-Fructofuranosyl-fructofuranose was much more abundant in apple (15.9 and 26.5 mg/100 g) than in peach cultivars (0.7 and 1.1 mg/100 g). The compound tentatively assigned as cellobitol was detected in higher concentrations in peach (10.6-19.1 mg/100 g) than in apple (1.8-4.3 mg/100 g). Additionally, maltitol, at a concentration between 1.7 and 3.0 mg/100 g, was found only in peach cultivars. Regarding the unidentified compounds, fructofuranosyl-fructofuranose-1 was detected only in apple cultivars (2.2-4.9 mg/100 g). The same occurred for fructofuranosyl-fructofuranose-2 (3.0-

4.6 mg/100 g) and pyranosyl-pyranose-2 (13.2-15.3 mg/100 g), corresponding to chromatographic peaks 16 and 17, respectively, and for pyranosyl-pyranosyl alcohol (11.2-19.2 mg/100 g), corresponding to chromatographic peak 18, which was not present in the Royal Gala apple cultivar. Fructofuranosyl-fructofuranose-3 was present in apple and peach cultivars at 8.7-12.9 mg/100 g and 4.7-6.1 mg/100 g, respectively. In contrast, pyranosyl-pyranose-1 presented major concentrations in peach cultivars (5.4-17.9 mg/100 g), whereas it was undetected in apple. Finally, a one-way ANOVA test was performed to assess possible compositional differences between fruit cultivars and varieties. In this regard, differences were observed between apple and peach with $\alpha < 0.05$, while no significant differences were detected within cultivars of the same fruit.

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3.2. Injection-port derivatization coupled to GC-MS or GC-MS/MS for the analysis of several metabolomics compounds in fruit and fruit juice samples

This Chapter is focused on the use of injection-port derivatization as an on-line step [1] to perform the analysis of several classes of high-boiling compounds which require conversion into trimethylsilyl derivatives to increase their volatility and thermostability to be suitable for their analysis by GC. Traditionally, this reaction has been performed off-line following the evaporation of the extract [2-4], which results in higher volumes of derivatization reagent needed, higher sample preparation time, and possible analyte degradation.

The first section reports by the first time the analysis of glycosylated and non-glycosylated polyphenols in fruits and fruit juice samples employing gas chromatography. Traditionally, this group of compounds has been analysed by HPLC [5]. However, employing injection-port derivatization coupled to GC-MS/MS allows the targeted analysis of polyphenols at good LOD. In addition, during sample preparation a C18 SPE clean-up has been tested to reduce matrix effects, as the extraction of polar compounds in fruit samples includes sugars, and sugar alcohols which are present at high concentrations and may compete with the compounds under study during the silylation reaction [6].

The second section deals with the determination of HMF and patulin in commercial fruit juices. These compounds are of major importance in juice industry as they can be considered as markers of the quality of a fruit-derived product [7]. Prior to analysis, juice samples have been extracted with ethyl acetate as previously reported [8]. With the proposed methodology existing GC methods [3, 9] have been improved in terms of time and derivatization reagent consumption. Moreover, the use of MS/MS is very helpful to achieve lower LOD without the need of sample pre-concentration.

Finally, the last section of the Chapter is focused on the determination of the lipophilic fraction of fruit juices, in a targeted analysis which includes fatty acids (4), fatty alcohols (4), phytosterols (3) and triterpenes (3). In this case, a liquid-phase microextraction technique, namely DLLME [10] has been optimized employing $CHCl_3$ and acetone as extraction and dispersive solvent, respectively. After extraction, the sample can be directly submitted to analysis employing injection-port trimethylsilylation. In this study, compounds have been detected with mass spectrometry working in SIM mode instead of employing MS/MS due to the latter resulted in high fragmentation and low abundancy of daughter ions.

The results obtained in this Chapter have been published in *Food Chemistry,* 2016, **204,** 210-217 and *Journal of Chromatography A,* 2016, **1453,** 99-104 and submitted to *Journal of Chromatography A*.

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3.2.1. Injection-port derivatization coupled to GC-MS/MS for the analysis of glycosylated and non-glycosylated polyphenols in fruit samples

INJECTION-PORT DERIVATIZATION COUPLED TO GC-MS/MS FOR THE ANALYSIS OF GLYCOSYLATED AND NON-GLYCOSYLATED POLYPHENOLS IN FRUIT SAMPLES

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Abstract

Polyphenols, including glycosylated polyphenols, were analysed via a procedure based on injection-port derivatization coupled to gas chromatography-tandem mass spectrometry (GC-MS/MS). The polyphenols in lyophilized fruit samples were extracted with an acidified MeOH mixture assisted by ultrasound. Samples were dried under vacuum, and carbonyl groups were protected with methoxylamine. Free hydroxyl groups were subsequently silylated in-port. Mass fragmentations of 17 polyphenol and glycosylated polyphenol standards were examined using Multiple Reaction Monitoring (MRM) as the acquisition mode. Furthermore, in-port derivatization was optimized in terms of optimal injection port temperature, derivatization time and sample: *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) volume ratio. A C18 solid-phase-extraction clean-up method was used to reduce matrix effects and injection liner degradation. Using this clean-up method, recoveries for samples spiked at 1 and 10 µg/g ranged from 52% to 98%, depending on the chemical compound. Finally, the method was applied to real fruit samples containing the target compounds. The complete chromatographic runtime was 15 min, which is faster than reported for recent HPLC methods able to analyse similar compounds.

Keywords*: glycosylated polyphenols, non-glycosylated polyphenols, in-port derivatization, GC-MS/MS, fruit samples.*

1. Introduction

Polyphenols, a type of diet-derived anti-oxidant, have received considerable public attention due to their protective effects against cancer and cardiovascular and age-related diseases (Cao, et al., 2008). Classified into anthocyanins, flavones, isoflavones, flavanones, flavonols, and flavanols (Tsao & Yang, 2003), these compounds are found not only in natural food sources such as fruits (Ignat, Volf, & Popa, 2011), but also in agro-industrial by-products (Delpino-Rius, Eras, Vilaró, Cubero, Balcells, & Canela-Garayoa, 2015) and in beverages, such as tea (Ding, Yang, & Xiao, 1999) and wine (Río Segade, Orriols, Giacosa, & Rolle, 2011).

Polyphenols have been extracted from fruit samples by means of several techniques depending on the sample; most of these include the use of a slightly acidic mixture of aqueous-organic solvents. The extraction is usually assisted by microwave or ultrasound (Picó, 2013). Recently, micro-extraction techniques, which require lower amounts of solvent, have also been used for this purpose (Nerín, Salafranca, Aznar, & Batlle, 2009).

After extraction, the various groups of phenols are commonly analysed by reversed-phase HPLC using a C18 column and UV-vis diode array detector (DAD) (Schieber, Keller, & Carle, 2001). Mass and tandem mass spectrometry play an important role, especially for identification purposes (Campillo, Viñas, Férez-Melgarejo, & Hernández-Córdoba, 2015 and Malec, Le Quéré, Sotin, Kolodziejczyk, Bauduin, & Guyot, 2014). Although HPLC is the primary technique used for the analysis of polyphenols, several studies refer to the analysis of flavonoid aglycones by gas chromatography using silylation to convert the analytes into volatiles (Nolvachai & Marriott, 2013). Examples of this type of analysis can be found using on-column injection (Vinciguerra, Luna, Bistoni, & Zollo, 2003), analysis of polyphenols in apple pomace (Tao, Sun, Chen, Li, Wang, & Sun, 2014) and apple juice (Loots, van der Westhuizen, & Jerling, 2006). Flavonoid aglycones have also been explored in model systems and citrus fruits (Füzfai & Molnár-Perl, 2007) by means of a prior oximation step to obtain a better response, particularly for anthocyanins and apple (Rudell, Mattheis, & Curry, 2008). In addition, a few studies have attempted to analyse flavonoid glycosides as trimethylsilyl (TMS) derivatives using high temperature chromatography (dos Santos Pereira, Costa Padilha, & Radler de Aquino Neto, 2004). However, these efforts were only qualitative.

Derivatization is often carried out off-line after extraction; however, the possibility has emerged of performing this derivatization on-line, thereby reducing time-consuming sample processing steps, decreasing the amount of reagents, and increasing the analytic speed and efficiency (Docherty & Ziemann, 2001). Among these alternative approaches, on-line processes involving the introduction of the sample and the derivatization reagent directly into the hot GC inlet are known as inlet-based or in-port derivatizations. In this procedure, the derivatization occurs in the gas-phase (Bizkarguenaga, et al., 2013). The sample and the derivatization reagent can be injected separately, either by first manually

injecting the sample or the derivatization reagent (Viñas, Martínez-Castillo, Campillo, & Hernández-Córdoba, 2011), requiring the presence of the analyst to start each analysis, or simultaneously, using a software controlled sandwich injection which fills the syringe with both the sample and the derivatization reagent, allowing an air gap between them. The latter is expected to give better results in terms of repeatability and automation of the analytical sequence.

The aim of this work was to develop an injection-port method using a GC-MS/MS instrument, with derivatization performed using an automated sandwich injection of the methoximated sample and the derivatization reagent, namely *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA). In addition, Multiple Reaction Monitoring (MRM) was used for mass acquisition, thus allowing an improvement of the limits of detection. This enhancement is especially useful in the case of glycosylated polyphenols as the derivatization yields of these compounds are generally low, thereby causing lower analyte response. Moreover, the use of electron ionization (EI) as the GC ionization source could provide a mass spectrum with more fragments, which could be a useful tool for identification purposes. The applicability of this method is demonstrated via the analysis of distinct samples drawn from fruit origins, known to be important sources of polyphenols. To the best of our knowledge, this report describes the first time that a method using GC has been used to analyse polyphenols and glycosylated polyphenols in a single analysis, thus broadening the field of GC applications into analyses traditionally performed by LC. In addition, the chromatographic run time is much faster than current LC methods, requiring only 15 min.

2. Material and Methods

2.1. Reagents, solvents, and phenolic standards

N-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) and methoxylamine hydrochloride (MEOX) were purchased from Sigma-Aldrich (Buchs, Switzerland).

Methanol (MeOH), acetone (HPLC grade purity), ethyl acetate (EtOAc), and pyridine were supplied by J.T. Baker (Deventer, The Netherlands), and water was purified in a Milli-Q system from Millipore (Bedford, MA, USA). Ascorbic acid was purchased from Acros (Pittsburgh, PA, USA) and glacial acetic acid (HAcO) from Panreac (Barcelona, Spain).

Standards of phenolic compounds were supplied as follows: (+)-catechin, (−)-epicatechin, procyanidin B1, procyanidin B2, quercetin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-rutinoside, kaempferol-3-*O*-rutinoside, isorhamnetin-3-*O*-rutinoside, epigallocatechin gallate, cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside by Extrasynthèse (Genay, France), and phloretin-2′-*O*-β-glucoside and 5′-caffeoylquinic acid

by Sigma-Aldrich Chemie (Steinheim, Germany). Standard stock solutions of 100 µg/mL of phenolic compounds were prepared in MeOH and stored at −80 °C in amber glass vials. Working solutions of 50 and 10 µg/mL were prepared from stock solutions by sampling an aliquot and diluting as necessary with MeOH.

A C18 SepPak® cartridge (400 mg packing, Waters, Milford, MA, USA) sorbent was used for solid-phase extractions (SPEs). A Visiprep SPE vacuum manifold from Supelco (Bellefonte, PA, USA) was used to process up to 12 SPE tubes simultaneously.

2.2. Instrumentation

The GC-MS/MS analyses were performed with an Agilent 7890 GC (Agilent Technologies, Palo Alto, CA, USA) with a multimode injector and a splitless liner containing a piece of glass wool. A fused silica high-temperature capillary column (J&W DB-1HT, 15 m × 0.32 mm i.d.; 0.10 µm film thickness) from Agilent was used at constant pressure. The detector was an Agilent 7000B triple quadrupole mass spectrometer with an inert EI ion source. The mass spectrometer worked in MRM mode with the EI ionization source at 70 eV. Helium with a purity of 99.9999% was used as both the carrier and quenching gas, and nitrogen with a purity of 99.999% as the collision gas, both supplied by Air Liquide (Madrid, Spain).

For control and data analysis, Agilent Mass Hunter B.04.00 software was used.

2.3. Samples

Randomly chosen Golden Delicious and Royal Gala apples, Blanquilla pears, and red plums were purchased from a local market (approximately 1 kg of each). In addition, processed foodstuffs of fruit origin, namely apple juice concentrate, natural peach juice, apple/peach juice, raspberry jam, and cranberry juice were supplied by local industries. Fruits were homogenized in a blender (Grindomix GM 200; Retsch, Haan, Germany) at 5000 rpm for 2 min, and ascorbic acid (~ 10 g/kg) was added to prevent oxidation. Samples were immediately frozen at −80 °C and lyophilized at −50 °C and 1.1 Pa for 24 h in a Cryodos-50 lyophilizer (Telstar, Terrassa, Spain). Processed foodstuff samples were frozen at −80 ºC before being lyophilized. Finally, the lyophilized samples were powdered and stored at −20 ºC until analysis.

2.4. Analytical procedure

Approximately 100 mg of each of the lyophilized samples was placed into 15-mL polypropylene tubes. Subsequently, 2 mL of a H_2O-methanol (20:80) solution acidified with 1% of HAcO was added to each tube. The mixture was subjected to an ultrasonic bath (ATU APM40-2LCD; Madrid, Spain) for 10 min followed by 20 min of vortex agitation and centrifugation at 1400×*g* for 10 min (Hettich Eppendorf Centrifuge MIKRO 22 R; Tuttlingen, Germany). A 1 mL volume of the extract was made up with 3 mL of deionized H_2O before SPE clean-up.

The C18 classic SepPak cartridge was first conditioned with 3 mL of methanol followed by 2 mL of H_2O (1% HAcO, v/v). The sample extract was then applied to the cartridge. Co-extracted substances (e.g., sugars and organic acids) were rinsed from the sorbent with H_2O (acidified at 1%, v/v with HAcO). Subsequently, the cartridge was eluted with 1.5 mL of methanol (1% HAcO, v/v) followed by 0.5 mL of EtAcO. The solvents were evaporated under reduced pressure at room temperature using a SpeedVac (Thermo, Asheville, NC, USA). The residue was dissolved in 300 µl of a solution of MEOX in pyridine (20 mg/mL) and incubated at 45 ºC for 1 h in a ThermoMixer (Eppendorf AG, Hamburg, Germany). Prior to injection into the gas chromatograph, the methoximated sample was placed in a chromatography vial containing a glass insert.

Sandwich injection of the sample and the derivatization reagent (MSTFA) in a ratio of 2:3 µl was carried out in splitless mode applying an inlet temperature program as follows: 100 ºC (held for 3 min), then increased to 320 ºC at 250 ºC/min. The GC oven temperature was programmed as follows: 70 ºC (held for 3 min), then increased to 270 ºC at 50 ºC/min, and then to 340 ºC at 10 ºC/min (held for 1 min) at a constant pressure of 10.31 psi. A 5-min backflush using a restrictor (0.7 m x 150 µm) inert capillary column at 340 ºC and 60 psi was programmed after each run to eliminate the compounds retained in the chromatographic column. These compounds result from the incomplete derivatization of some of the low volatility analytes. Moreover, the vial cap of the derivatization reagent was replaced every 10 injections to prevent contamination from the vial septum.

The temperatures of the ion source and the transfer line were 250 ºC and 300 ºC, respectively. An MRM method was created keeping the temperature of both quadrupoles at 150 ºC. Two transitions were monitored for each analyte, the first for quantification and the second for confirmation. Table 1 shows the selected mass spectrometer conditions. The resolution was adjusted to 1.0 Da for quadrupoles 1 and 3. The solvent delay was 5 min.

3. Results and Discussion

3.1. Optimization of the chromatographic and MS/MS conditions

The chromatographic conditions were optimized using a standard mixture to achieve the efficient separation of the 17 target compounds (see conditions in section 2.2) in a 15 min run—shorter than current HPLC methods (Díaz-García, Obón, Castellar, Collado, & Alacid, 2013, Fischer, Carle, & Kammerer, 2011, Castellar, Collado, & Alacid, 2013 and Fischer, Carle, & Kammerer, 2011). Quantitation parameters for all compounds are listed in Table 1.

Table 1: GC-MS/MS retention time and selected transitions for the target polyphenols.

Compound	RT (min)	Precursor ions (*m/z*)	Product ions (*m/z*)[a]	Collision energy (eV)[a]
(-)-Epicatechin	7.82	368	249, 265	20
(+)-Catechin	7.89	368	249, 265	20
5-Caffeoylquinic acid	8.23[b]/ 8.59	345	73, 255	35, 20
Quercetin	8.72	647	73, 575	60, 50
Cyanidin-3-*O*-galactoside	9.67	382	73, 355	20, 35
Cyanidin-3-*O*-glucoside	9.79	382	73, 355	20, 35
Phloretin 2′-*O*-glucoside	9.83	342, 547	327, 179	20, 20
Quercetin-3-*O*-galactoside	10.78	647	73, 576	60, 50
Quercetin-3-*O*-glucoside	10.89	647	73, 559	60, 60
Quercetin-3-*O*-rhamnoside	11.12	647	73, 560	60, 50
Epigallocatechin gallate	11.38	369	179, 281	35, 20
Cyanidin-3-*O*-rutinoside	12.38	382	73, 355	20, 35
Procyanidin-B2	12.54	368	249, 191	20, 20
Procyanidin-B1	12.60	368	249, 191	20, 20
Kaempferol-3-*O*-rutinoside	12.76	502	487, 415	20, 50
Quercetin-3-*O*-rutinoside	13.04	590, 575	575, 503	20, 50
Isorhamnetin-3-*O*-rutinoside	13.06	532	517, 487	20, 50

[a] Underlined values were used for quantification transitions.

[b] For 5-caffeoylquinic acid peaks corresponding to the two oximes formed during methoximation were observed.

Using the non-polar J&W DB-1HT column, the retention times of the target compounds increased with the number of TMS groups. This behaviour has been previously described in polyphenol studies, which have been analysed with similar non-polar columns (Gao, Williams, Woodman, & Marriott, 2010 and Koupai-Abyazani, Creaser, & Stephenson, 1992). Hence, higher retention times were observed for the compounds with a disaccharide as the glycosidic unit versus those that had a monosaccharide unit for polyphenols with the same aglycone. Moreover, aglycones had lower retention times

compared with the former two. Those studies also reported the retention order of TMS silylated polyphenols with the same substituents as flavan-3-ol< chalcone< flavonone< isoflavone< flavonol< flavone. On this basis, the retention time of anthocyanins appears to be close to that of chalcones.

3.2. Optimization of in-port derivatization

Trimethylsilyl derivatives are routinely used in GC to increase the volatility and thermal stability of organic compounds carrying hydroxyl groups. In this study, a prior methoximation of the dry sample was performed in order to protect the carbonyl groups—present in many of the structures—and to enhance the derivatization yield of the compounds. Moreover, the aprotic nature of pyridine, as a solvent that solubilizes derivatives, protects the target analytes against hydrolysis. EtAcO and hexane were also tested as alternatives to pyridine. The response obtained with these solvents diminished (data not shown).

Following methoximation, the silylation conditions of the methoximated extract prepared from a standard mixture of all target compounds were optimized in terms of time (purge off), temperature (Figure 1a), and sample volume/MSTFA ratio (Figure 1b). In this study, the sample and the derivatization reagent were sandwich injected simultaneously. Previous studies reported optimum temperatures approximately 200 ºC for the in-port derivatization of compounds, such as (+)-catechin and (-)-epicatechin (Viñas et al., 2011). However, at this temperature, a peak at the retention time of the quercetin aglycone was observed for glycosylated polyphenols, such as quercetin glycosides. This finding could be attributable to the breakage of the glycosylic bond, thus yielding a signal for quercetin. Therefore, temperatures between 70 ºC and 150 ºC were tested for the in-port derivatization of glycosylated polyphenols. This temperature range did not affect the method performance, as shown by a high response for aglycones. The best results were obtained at 100 ºC. The derivatization time ranged between 0.5 and 5 min, yielding maximum performance at 3 min (Figure 1a). Using these optimum conditions, the ratio of methoximated extract versus MSTFA volume was optimized using 1:1, 1:2, 2:1, 2:3 and 3:2 volume ratios. Similar results were obtained for 2:1 and 2:3 ratios using standards (Figure 1b). Analysis of variance (ANOVA) showed that the most significant parameters in the optimization of in-port derivatization at a 95% confidence level were injection temperature and sample:derivatization reagent ratio. As it is expected that the matrix may play a role in the optimum ratio, both conditions were further studied in real sample matrices of the fruits under study.

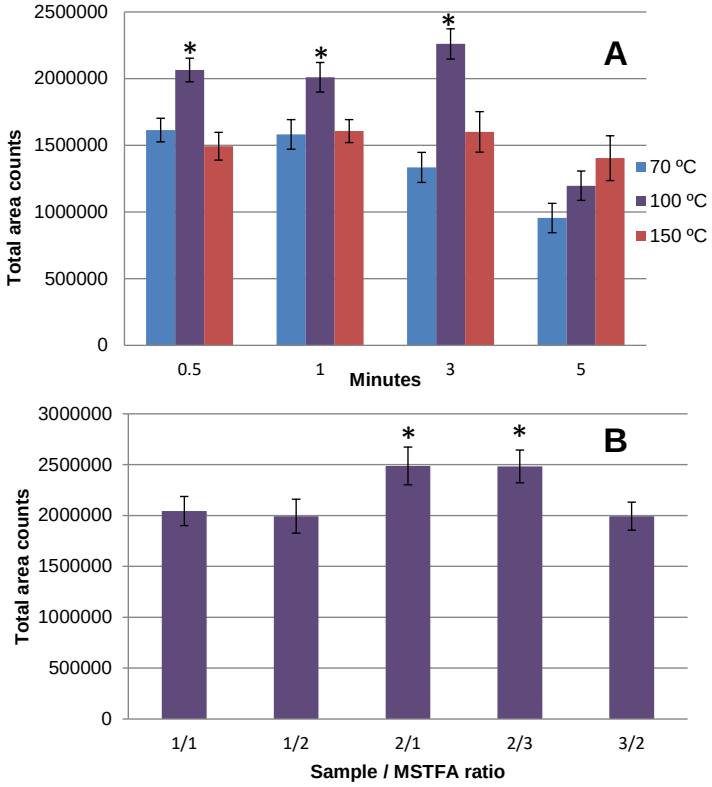

Figure 1: Optimization of in-port derivatization in terms of: A) temperature and time and B) sample:MSTFA volume ratio. *: shows conditions found to be statistically different (95% confidence level).

Sample matrices of different origin were spiked with all the target compounds at a concentration between 1 and 10 µg/mL and injected into the GC system using the two selected ratios for standards, namely sample:MSTFA volume ratios of 2:1 and 2:3. Figure 2 shows that the second condition led to a marked improvement in the detection of polyphenols. This enhancement was especially high for most of the compounds in raspberry jam. This could be attributable to this matrix containing a higher amount of sugars, which interfere with the derivatization reaction of the target analytes. On the other hand, a considerable decrease in response for phloretin 2′-*O*-glucoside, cyanidin-3-*O*-glucoside, and cyanidin-3-*O*-rutinoside was observed in apple samples compared with the other two fruit samples, likely due to the higher content of organic acids in this matrix. Although a decrease was noticed for these three compounds in a certain matrix, in general a considerable increase was observed in the responses for most of the

compounds when using a 2:3 ratio. This finding may be attributable to the fact that fruit matrices contain a high concentration of co-extractives, such as sugars and organic acids, which reduce the derivatization efficiency of polyphenols. Consequently, a sample:MSTFA volume ratio of 2:3 was used for further analyses.

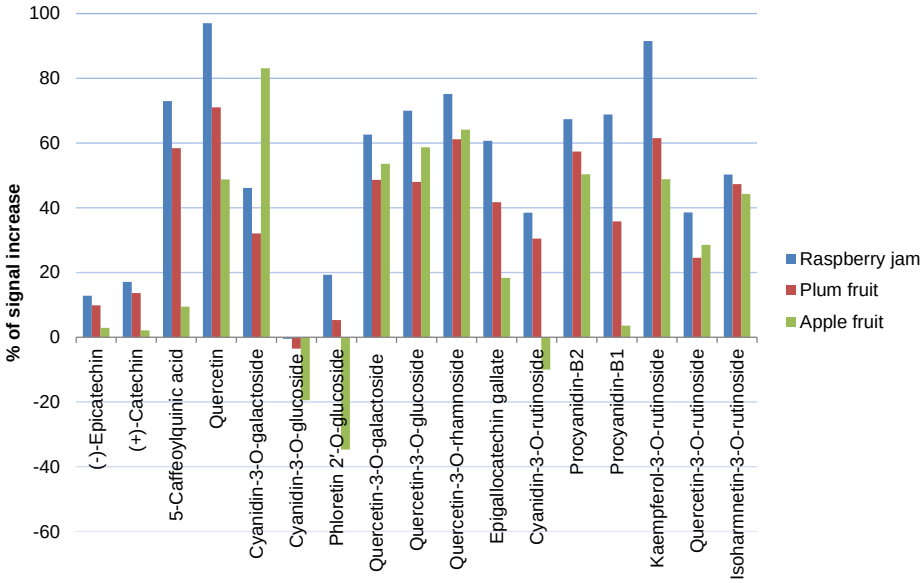

Figure 2: Effect of the sample:MSTFA ratio on response variation for each of the target compounds in three matrices spiked with standards. Data are presented as relative percentage between responses resulting from 2:3 versus 2:1 ratio.

3.3. Method performance

The performance parameters of the GC-MS/MS method for the optimized conditions described in sections 3.1 and 3.2 were evaluated in terms of LOD, LOQ, and intra-and inter-day repeatability (expressed as relative standard deviation), correlation coefficient (r) and linear range as summarized in Table 2. In this regard, LOD and LOQ were calculated as the concentrations giving S/N=3 and S/N=10, respectively, for standard solutions (due to the impossibility of spiking blank fruit samples, as they are natural sources of the target compounds). Because of this, instrumental limits were lower for low molecular weight compounds, namely aglycones, with LODs between 6-30 ng/mL and LOQs between 20-100 ng/mL, increasing for those with a monosaccharide as a glycoside unit and for those with a disaccharide as a glycoside unit (LOD <240, LOQ <800

ng/mL), which in the case of the studied target compounds was rutinose. The higher LOD and LOQ values obtained for higher molecular weight compounds could be attributed to a lower derivatization yield due to the high number of hydroxyl groups present in these molecules, with their consequent steric hindrance. We have previously assayed off-line derivatization for glycosylated polyphenols, observing similar behaviour (data not shown). The LODs for aglycones were very similar to those reported in modern HPLC-DAD methodologies (≤20 ng/mL) (Abad-García, Berrueta, López-Márquez, Crespo-Ferrer, Gallo, & Vicente, 2007) and better than observed with other previously published methods (Tsao & Yang, 2003). Although the efficiency of the derivatization decreases with the molecular weight, the described methodology showed higher LODs than HPLC for glycosylated polyphenols, which for HPLC are approximately 30 ng/mL using modern methods, and very similar or even better than those reported by Tsao and Yang (2003). Repeatability was studied at two concentration levels of the methoximated extract (1 µg/mL and 5 µg/mL). Upright %RSD values were obtained for intra-and inter-day repeatability, ranging from 3% to 12% and from 5% to 18%, respectively. Repeatability values are better for aglycones (<9%), and they generally increase with the molecular weight due to a loss of the derivatization efficiency. Correlation coefficient values ranged from 0.973 to 0.999. The linear range in which calibration curves were studied showed the same behaviour as reported for the limits of detection, where lower molecular weight species, namely (-)-epicatechin, (+)-catechin and 5-caffeoylquinic acid allowed a greater linear range than glycosylated species.

Table 2: Quality parameters for the analysis of polyphenol standards.

Compound	LOD (ng/mL)	LOQ (ng/mL)	Intra-day repeatability (%RSD, n=5)		Inter-day repeatability (%RSD, n=5)		Correlation coefficient	Linear range
			1 µg/mL	5 µg/mL	1 µg/mL	5 µg/mL	(r)	(µg/mL)
(-)-Epicatechin	6	20	7	5	5	7	0.995	0.020-5.1
(+)-Catechin	6	20	6	4	7	6	0.995	0.020-5.9
5-Caffeoylquinic acid	15	50	8	3	9	8	0.994	0.050-6.8
Quercetin	30	100	7	4	9	7	0.999	0.163-10.4
Cyanidin-3-*O*-galactoside	30	100	8	6	12	10	0.982	0.114-14.4
Cyanidin-3-*O*-glucoside	30	100	9	5	14	11	0.986	0.114-17.2
Phloretin 2′-*O*-glucoside	30	100	7	4	10	8	0.990	0.114-11.2
Quercetin-3-*O*-galactoside	240	800	10	6	12	10	0.992	0.866-13.8
Quercetin-3-*O*-glucoside	240	800	11	7	15	10	0.980	0.772-12.4
Quercetin-3-*O*-rhamnoside	240	800	11	6	15	10	0.998	0.833-13.4
Epigallocatechin gallate	100	300	8	4	11	9	0.973	0.174-11.2
Cyanidin-3-*O*-rutinoside	30	100	8	4	12	9	0.988	0.114-7.2
Procyanidin-B2	15	50	7	5	9	9	0.990	0.026-6.8
Procyanidin-B1	15	50	7	3	8	7	0.992	0.043-5.6
Kaempferol-3-*O*-rutinoside	180	600	10	6	12	10	0.997	0.452-13.8
Quercetin-3-*O*-rutinoside	240	800	12	7	18	14	0.982	0.864-13.8
Isorhamnetin-3-*O*-rutinoside	100	300	9	6	11	8	0.997	0.362-8.4

3.4. Matrix effects

In gas chromatography, matrix effects may occur in the injection port, where the derivatization reaction takes place. Furthermore, the analytes of the matrix include many co-extractives, mainly carbohydrates and organic acids, which compete for the derivatization reagent. Matrix effects were therefore studied in order to determine the feasibility of using an external standard calibration curve to quantify the analytes. The matrix effects were assessed in three matrices of distinct origin (apple fruit, red plum fruit, and raspberry jam) and were calculated by comparing the signal response obtained when spiking a sample after extraction (at 1 µg/mL for aglycones and at 10 µg/mL for glycosylated polyphenols) with the signal response obtained from a standard solution at the same concentration (Eq. 1).

$$\%\,\mathrm{ME} = \left(\frac{\text{Area post extraction spiked sample}}{\text{Area of standard}} - 1\right) \times 100 \qquad (1)$$

A non-spiked sample was also analysed for each of the matrices in order to subtract the signal produced for compounds already present in the sample. For all the matrices, the signal considerably decreased for compounds that gave a lower response, namely glycosylated flavonols, anthocyanins, and procyanidin dimers, with the decrease being especially noticeable for the first two. This can be explained by the fact that although an increase in the ratio of derivatization reagent in the injection port gave an increase in the response, it was not enough to achieve a response equivalent to the same concentration of the compounds in the standard solution. In contrast, (-)-epicatechin, (+)-catechin, and 5-caffeoylquinic acid showed the opposite behaviour, giving a slight signal enhancement (<28%). In general, enhancement could be attributed to the presence of co-extractives, which mask the active sites in the chromatographic system, resulting in lower adsorption of the analytes (generally in the liner) resulting in signal enhancement.

Moreover, the reproducibility of the derivatization reaction in the different matrices over time under these conditions was not consistent, most likely because the injection of a large amount of matrix components caused gradual accumulation of non-volatile components in the GC system, resulting in the formation of new active sites and a gradual decrease in analyte response (Rahman, Abd El-Aty, & Shim, 2013). According to Schenck et al., two opposing phenomena should be considered when studying matrix effects in GC. One is the degree of enhancement of the analyte response after repeated injections. The second is decreases in the responses as a result of a dirty injection liner (Schenck & Lehotay, 2000). Considering these concerns, a clean-up step to reduce these effects was studied.

3.4.1. SPE clean-up

In order to reduce the matrix effects and simultaneously improve the reproducibility of sample analysis, a clean-up step using a C18 SepPak was introduced into the analytical method (Wrolstad, et al., 2005). After being conditioned, the column was loaded with the sample and washed with aqueous acid solution to remove carbohydrates and organic acids. Finally, polyphenols were eluted with 1.5 mL of methanol (acidified at 1%, v/v with HAcO). Note that in some studies a 0.1% HCl solution is used to elute polyphenols. The acid tends to stabilize polyphenols, especially anthocyanins; however, it can also cause acid hydrolysis during concentration to dryness (Wrolstad et al., 2005), which is an essential step in GC analyses of the nature reported here. Consequently, a weaker acid, HAcO, was used. To assess the suitability of performing a clean-up step with a C18 cartridge, three matrices under study (apple fruit, red plum fruit, and raspberry jam) were spiked before performing the SPE and the areas obtained for the target polyphenols compared with those obtained for standard mixtures at the same concentration. Table 3 shows that after applying the SPE clean-up, the signal improved for all analytes (except cyanidin-3-*O*-rutinoside, which showed a slight decrease) compared to the response obtained without this clean-up. Matrix effects for the other compounds showed an enhancement generally below 20%, except for phloretin 2′-*O*-glucoside, which increased to 60%. Given that matrix effects were highly reduced and controlled with the use of SPE, calibration by external calibration curve was used because good correlation values were obtained for most of the analytes (Table 2). In addition, the alternatives to this calibration would have been standard addition, which is time-consuming and labour intensive, or the use of an internal standard, which was not suitable because the derivatization and analytical performance of each of the target compounds is very different.

Table 3: M.E (%) and recoveries (%) obtained in three matrices spiked at 1 µg/mg for aglycones and at 10 µg/mg for glycosylated polyphenols.

Compound	M.E. (%)		Recoveries (%)	
	without SPE clean-up	with SPE clean-up	2 mL MeOH	1,5 mL MeOH + 0,5 mL EtOAc
(-)-Epicatechin	10	13	92	96
(+)-Catechin	11	14	90	94
5-Caffeoylquinic acid	20	30	49	52
Quercetin	-36	30	66	84
Cyanidin-3-*O*-galactoside	-70	19	78	83
Cyanidin-3-*O*-glucoside	-70	23	76	77
Phloretin 2'-*O*-glucoside	-65	63	80	98
Quercetin-3-*O*-galactoside	-83	32	68	80
Quercetin-3-*O*-glucoside	-72	23	72	83
Quercetin-3-*O*-rhamnoside	-67	23	75	88
Epigallocatechin gallate	-51	-9	71	91
Cyanidin-3-*O*-rutinoside	-86	10	80	85
Procyanidin-B2	-19	8	88	88
Procyanidin-B1	-20	6	76	82
Kaempferol-3-*O*-rutinoside	-37	20	75	86
Quercetin-3-*O*-rutinoside	-47	13	78	89
Isorhamnetin-3-*O*-rutinoside	-52	12	80	92

% RSD (n=3) <25%.

Recoveries were assessed in three spiked matrices (at 1 µg/mg for aglycones and at 10 µg/mg for glycosylated polyphenols) applying the C18 SPE clean-up. Moreover, two elution solvent combinations were studied to enhance recoveries after the clean-up (Table 3). The main drawback of this clean-up step was that 5-caffeoylquinic acid showed

low recoveries (approximately 50%) as it was partially washed off the column with H_2O. Recoveries for the other target compounds ranged from 66 to 92% and from 77 to 96% when using methanol and methanol-EtOAc, respectively. Recoveries generally improved when the elution with acidified methanol was followed by EtOAc. This improvement was especially noticeable for the most apolar compounds, such as flavonols and flavonol glycosides, whereas anthocyanins and flavan-3-ols showed little improvement. Consequently, the combination of 1.5 mL of MeOH and 0.5 mL of EtOAc was selected for further analyses.

3.5. Application to samples

The optimized methodology was applied to determine 17 target polyphenols in Golden Delicious and Royal Gala apples, Blanquilla pears, and plum fruit as well as in processed foods of fruit origin, namely, apple juice concentrate, natural peach juice from the Clingstone cultivar, a mixture of apple and peach juice, raspberry jam, and cranberry juice (Table 4). All samples were analysed in triplicate.

All of the samples contained the flavan-3-ols (-)-epicatechin and (+)-catechin, which were present at concentrations ranging from 1.3 to 2413.1 µg/g. A strong relationship was observed between these compounds and their corresponding dimers, namely procyanidin B2 and procyanidin B1, as previously reported in peach (Scordino, Sabatino, Muratore, Belligno, & Gagliano, 2012) and apple (Tsao, Yang, Young, & Zhu, 2003) samples. In addition, procyanidin B2 was the compound found at the highest concentration (5187.3 µg/g) in a plum fruit sample. Although 5-caffeoylquinic acid gave lower recoveries, most of the samples showed high concentrations of this compound (1.9-4350.4 µg/g). Variable concentrations of quercetin glycosides were detected only in apple and plum fruit, raspberry jam, and cranberry juice. In samples containing flavonol glycosides, aglycone quercetin was consistently present, although generally at very low concentrations (<5.4 µg/g). The other flavonols, namely isorhamnetin-3-*O*-rutinoside and kaempferol-3-*O*-rutinoside, were detected only in peach juice products, at low concentrations. The dihydrochalcone phloretin 2′-*O*-glucoside was found only in apple-based products, both fresh fruit and juice. This result is consistent with that reported by several authors (Spanos, Wrolstad, & Heatherbell, 1990). Anthocyanins were found only in samples which had been previously reported in the literature, such as raspberry jam, cranberry juice, plum fruit, peach juice, and Royal Gala apple (Welch, Wu, & Simon, 2008). The content fluctuated noticeably, ranging from traces up to 58.5 µg/g and from 0.1 to 337.4 µg/g for cyanidin-3-*O*-galactoside and cyanidin-3-*O*-glucoside, respectively. Cyanidin-3-*O*-rutinoside was detected only in plum fruit but at a concentration of 57.9 µg/g. These results support the theory that food processing causes the degradation of polyphenols (Kahle, Kraus, & Richling, 2005). In this regard, the content of flavonols and flavan-3-ols decreased considerably between fresh apple fruit and an apple juice concentrate. In the peach juice, which was prepared by squeezing rather than from

concentrate, such as the apple juice, the polyphenol content was higher, giving notable concentrations of flavan-3-ols, flavonols, and anthocyanins.

Table 4: Sample analysis expressed as µg/g (dry weight).

Compound	Golden delicious	Royal gala	Pear	Plum fruit	Apple juice	Peach juice	Apple/ peach juice	Raspberry jam	Cranberry juice
(-)-Epicatechin	748.7 ± 3.0	962.7 ± 19.3	2.4 ± 0.5	2413.1 ± 48.3	4.6 ± 0.8	14.6 ± 1.9	7.7 ± 1.5	153.5 ± 5.2	58.5 ± 8.5
(+)-Catechin	27.2 ± 4.1	52.8 ± 4.2	<LOQ	277.3 ± 13.9	1.3 ± 0.3	177.0 ± 22.8	86.4 ± 8.6	4.0 ± 1.2	3.0 ± 0.6
5-Caffeoylquinic acid	3763.1 ± 75.3	4350.4 ± 130.5	20.5 ± 0.6	127.6 ± 16.6	868.7 ± 29.5	3589.8 ± 71.8	2514.7 ± 186.1	1.9 ± 0.5	131.3 ± 21.5
Quercetin	<LOQ	5.4 ± 1.6	<LOQ	6.9 ± 0.7	<LOQ	<LOQ	<LOQ	<LOQ	39.1 ± 6.3
Cyanidin-3-*O*-galactoside	n.d.	53.5 ± 2.7	n.d.	89.6 ± 4.7	n.d.	n.d.	<LOQ	13.0 ± 2.1	29.2 ± 10.8
Cyanidin-3-*O*-glucoside	n.d.	n.d.	n.d.	337.4 ± 32.1	n.d.	1.5 ± 0.2	1.1 ± 0.3	33.2 ± 4.2	n.d.
Phloretin 2'-*O*-glucoside	455.2 ± 45.5	113.8 ± 13.7	n.d.	n.d.	34.7 ± 7.3	n.d.	7.3 ± 2.0	n.d.	n.d.
Quercetin-3-*O*-galactoside	2.8 ± 0.7	58.2 ± 3.7	n.d.	3.4 ± 0.7	n.d.	n.d.	<LOQ	n.d.	58.5 ± 7.4
Quercetin-3-*O*-glucoside	n.d.	<LOQ	n.d.	<LOQ	n.d.	n.d.	<LOQ	<LOQ	<LOQ
Quercetin-3-*O*-rhamnoside	9.1 ± 0.39	20.6 ± 2.5	n.d.	47.5 ± 14.0	<LOQ	n.d.	<LOQ	3.3 ± 0.4	26.2 ± 4.4
Epigallocatechin gallate	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ	<LOQ	<LOQ	n.d.
Cyanidin-3-*O*-rutinoside	n.d.	n.d.	n.d.	152.3 ± 7.8	n.d.	n.d.	n.d.	n.d.	n.d.
Procyanidin-B2	985.6 ± 39.4	1024.4 ± 30.7	n.d.	5187.3 ± 544.7	<LOQ	490.1 ± 16.7	10.0 ± 1.4	n.d.	34.6 ± 7.6
Procyanidin-B1	268.3 ± 34.9	270.0 ± 43.2	n.d.	231.0 ± 37.2	n.d.	23.3 ± 2.8	317.2 ± 67.6	n.d.	n.d.
Kaempferol-3-*O*-rutinoside	n.d.	n.d.	n.d.	<LOQ	n.d.	4.3 ± 0.5	2.7 ± 67.6	n.d.	n.d.
Quercetin-3-*O*-rutinoside	n.d.	n.d.	n.d.	57.9 ± 3.1	n.d.	n.d.	n.d.	n.d.	n.d.
Isorhamnetin-3-*O*-rutinoside	n.d.	n.d.	n.d.	n.d.	n.d.	10.1 ± 1.0	4.7 ± 1.4	n.d.	n.d.

Values are mean ± standard deviation (n=3).

n.d.: not detected; <LOQ: detected but with a S/N<10.

4. Conclusions

An analytical method consisting of injection-port derivatization coupled to gas chromatography-tandem mass spectrometry was developed to determine 17 target polyphenols, including glycosylated polyphenols, in various fruit matrices. The chromatographic separation of the compounds was achieved in only 15 min, which is faster than reported for recent HPLC methods able to analyse similar compounds. Injection-port derivatization was optimized at 3 min and 100 ºC with a 2:3 sample:derivatization reagent ratio. LOD and LOQ were assessed for the target compounds, giving values below 240 and 800 ng/mL, respectively. Repeatability (%RSD at 1 µg/mL and 10 µg/mL, n=5) was below 18% for all the target compounds. In addition, a clean-up step with a C18 SPE cartridge was necessary to reduce matrix effects produced by the high abundance of sugars and organic acids co-extracted with the target compounds and to prevent the rapid deterioration of the injection liner. Finally, the method was applied to various fruit samples that are known sources of the target compounds. The polyphenol contents of the samples ranged from traces up to 5187.3 µg/g (procyanidin B2 in plum fruit). To summarize, this method offers a new and fast alternative to HPLC to analyse target polyphenols in several fruit samples, which is of great interest in food science.

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3.2.2. A rapid injection-port derivatization coupled to gas chromatography-tandem mass spectrometry method for the combined analysis of patulin and 5-hydroxymethylfurfural in fruit juices

A RAPID INJECTION-PORT DERIVATIZATION COUPLED TO GAS CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY METHOD FOR THE COMBINED ANALYSIS OF PATULIN AND 5-HYDROXYMETHYLFURFURAL IN FRUIT JUICES

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Abstract

A novel method consisting of injection-port derivatization coupled to gas chromatography tandem mass spectrometry is described. The method allows the rapid assessment of 5-hydroxymethylfurfural (HMF) and patulin content in apple and pear derivatives. The chromatographic separation of the compounds was achieved in a short chromatographic run (12.2 min) suitable for routine controls of these compounds in the fruit juice industry. The optimal conditions for the injection-port derivatization were at 270 ºC, 0.5 min purge-off, and a 1:2 sample:derivatization reagent ratio (v/v). These conditions represent an important saving in terms of derivatization reagent consumption and sample preparation time. Quality parameters were assessed for the target compounds, giving LOD of 0.7 and 1.6 µg/kg and LOQ of 2 and 5 for patulin and HMF, respectively. These values are below the maximum patulin concentration in food products intended for infants and young children. Repeatability (%RSD, n=5) was below 12% for both compounds. In addition, the method linearity ranged between 25 and 1000 µg/kg and between 5 and 192 µg/kg for HMF and patulin, respectively. Finally, the method was applied to study HMF and patulin content in various fruit juice samples.

Keywords: *injection-port derivatization, GC-MS/MS, HMF, patulin, fruit juices.*

1. Introduction

Patulin (4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one) is a mycotoxin produced by approximately 60 species of micro-organisms, including *Aspergillus, Penicillium, Mucor,* and *Fusarium* [1-3]. Some of these micro-organisms are responsible for the rotting of fruits like apples, pears, and cherries. Consequently, the presence of patulin in fruit derivatives is an indicator of the quality of the feedstock used in the manufacturing process [4]. Although the toxicity of patulin has been widely reviewed [5], no general consensus has been reached about its true degree of toxicity. Nevertheless, government agencies in the European Union have regulated the following maximum patulin concentration in food products intended for infants and young children: 50 µg/kg in juices; 25 µg/kg in solid apple products; and 10 µg/kg in apple products [6].

5-Hydroxymethylfurfural (HMF) is one of the main products of the Maillard reaction, which may occur during food processing and storage, particularly at high temperatures in carbohydrate-rich products. Moreover, HMF can also be produced during the acid-catalyzed dehydration of hexoses via 1,2 enolysation [7] or by glucosamine hydrolysis [8]. It is present naturally in products in which water coexists with monosaccharides in acid medium, such as balsamic vinegar and fruit juice [9].

In this context, analyses of patulin and HMF are now routine procedures for some widely consumed agro-food products, especially apple-derived products. These two compounds can be considered markers of the quality of a fruit-derived product [10]. The presence of patulin and HMF has commonly been determined by HPLC, either using a diode array detector (DAD) [4, 11-13] or a mass spectrum (MS) detector, the latter allowing a substantial increase in the selectivity of the analytical methods [14-17].

Patulin and HMF have also been determined independently by GC analysis of their trimethylsilyl ether, acetate or chloroacetate derivatives using either GC-FID [18, 19] or GC-MS in SIM mode, the latter allowing improved selectivity in complex matrices [20-24]. The derivatization process improves the otherwise low volatility of these two compounds, which have also been analyzed without this derivatization step, although analytical performance diminished [20, 25]. To the best of our knowledge, there is only one publication reporting the simultaneous GC analysis of patulin and HMF in apple juice in a qualitative rather than quantitative manner [22].

Derivatization is often performed off-line after the extraction of the sample. Off-line silylation procedures suffer from experimental errors such as loss of analyte through evaporation and re-suspension steps, contamination of samples during work-up, and the interference of moisture in the reaction system, since silylating reagents and the resulting derivatives are extremely sensitive to the presence of water. However, the possibility to perform this derivatization on-line has emerged [26, 27]. These approaches allow the reduction of time-consuming sample processing steps, a decrease in the amount of reagents required, and an increase in the speed and efficiency of the analysis. Inlet-based

or in-port derivatization is one of these alternative approaches. This on-line process involves introducing the sample and the derivatization reagent directly into the hot GC inlet, where the derivatization reaction takes place in the gas-phase [28]. The sample and the derivatization reagent can be injected separately. This can be achieved by first injecting the sample or the derivatization reagent manually [29], thus making the presence of the analyst inevitable in order to start each analysis. Alternatively, injection of the sample and reagent can be attained simultaneously by using a software-controlled sandwich injection, which fills the syringe with both the sample and the derivatization reagent, allowing an air gap between them. The latter approach is expected to give better results in terms of repeatability and automation of the analytical sequence.

Here we sought to develop a new method for the simultaneous analysis of patulin and HMF in fruit juice. The sample was initially extracted with ethyl acetate because of its accepted efficiency [4]. Secondly, the ethyl acetate solution was analyzed using injection-port derivatization and GC-MS/MS. Derivatization was optimized in terms of purge-off time, temperature, sample:derivatization reagent volume ratio, and MS/MS transitions selected for both compounds. The method circumvents the evaporation of the extract prior to derivatization, thus reducing both analyte degradation and sample preparation time. Furthermore, a cleanup step was discarded because the enhanced sensitivity and selectivity achieved with the triple quadrupole was considered to prevent both the concentration and purification of the sample.

2. Material and methods

2.1. Reagents, solvents, standard solutions and samples

N-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) was purchased from Sigma-Aldrich (Buchs, Switzerland), ethyl acetate (EtOAc) from J.T. Baker (Deventer, The Netherlands), anhydrous sodium sulfate from Acros (Pittsburgh, PA, USA), and sodium chloride from Panreac (Barcelona, Spain).

Stock standard solutions of patulin, 1000 µg/mL, and 5-(hydroxymethyl)-2-furaldehyde (HMF), 100 µg/mL, were prepared from the corresponding solid chemical reagents (Sigma Aldrich). Working solutions of 10 µg/mL and 5 µg/mL were prepared with EtOAc from consecutive dilutions of the stock solutions. All standard solutions were stored at -20 ºC and warmed to room temperature before using. In addition, the exact concentration of the patulin standard solution was determined to correct the possible losses of this compound by interaction with the glassware container [4]. A volume of 1 mL of patulin solution was evaporated to dryness under a stream of N_2. The residue was immediately dissolved in 20 mL of ethanol. The absorption spectrum was recorded between λ=250 nm and λ=350 nm in a 1-cm quartz glass cell in a spectrophotometer with ethanol in the reference path. The concentration of patulin was calculated using the following equation [30]:

$$p_m = \lambda_{max} \times m \times 100 / \varepsilon \times \delta$$

where p_m is the mass concentration of patulin in μg/mL; λ_{max} is the absorbance of patulin solution determined at λ=276 nm; m is the molecular mass of patulin (m=154 g/mol); ε (1460 m^2/mol in ethanol) is the molar absorptivity of a patulin solution at λ=276 nm; and δ is the path length of the quartz cell in cm (δ=1 cm).

Apple and pear juice concentrate samples from different production units were supplied by Zucasa (Fraga, Spain). Additional samples of commercial cloudy apple and pear juice were purchased from a local supermarket.

2.2. Instrumentation

The GC-MS and GC-MS/MS analyses were performed with an Agilent 7890 GC (Agilent Technologies, Palo Alto, CA, USA) with a multimode injector and a splitless liner containing a piece of glass wool. A fused silica high-temperature capillary column J&W DB-5MS (30 m × 0.25 mm i.d.; 0.25 μm film thickness) from Agilent was used at constant flow. The detector was an Agilent 7000B triple quadrupole mass spectrometer with inert EI ion source. The mass spectrometer worked in SIM or MRM mode with EI ionization source at 70 eV. Helium with a purity of 99.9999% was used as carrier gas and quenching gas, and nitrogen with a purity of 99.999% as collision gas, both supplied by Air Liquide (Madrid, Spain).

For control purposes and data analysis, the Agilent Mass Hunter B.06.00 software was used.

2.3. Analytical procedure

We placed 5 g of the homogenized sample into a 50-mL centrifugation tube. Subsequently, 10 mL of EtOAc was added. The mixture was vigorously shaken for 1 min by hand. Next, the tube was centrifuged for 5 min at 5000 rpm (Multi Reax; Heidolph, Schwabach, Germany). A volume of 1.5 mL of the upper layer was transferred into a 2-mL Eppendorf vial containing 100 mg of anhydrous sodium sulfate. The vial was manually shaken for 1 min and centrifuged for 3 min at 12,000 rpm (Hettich Eppendorf Centrifuge MIKRO 22 R; Germany). Finally, the organic phase was transferred to a crimp-cap vial for injection into the gas chromatograph.

Sandwich injections of the sample and the derivatization reagent (MSTFA) in various volume ratios were carried out in splitless mode between 230 to 290 ºC using a silanized glass insert containing a piece of glass wool with various purge-off time values. The gas chromatograph temperature was programmed as follows: 70 ºC (held for 1 min) to 320 ºC at 25 ºC/min (held for 2 min) at a constant flow regime of 1 mL/min. In addition, the cap of the vial containing the derivatization reagent was PTFE/Silicone/PTFE which

allows repeating injections and replaced every 20 injections to prevent contamination from the septum.

The temperatures of the ion source and the transfer line were 250 ºC and 300 ºC, respectively. The mass spectra detector operated in selected ion monitoring (SIM) mode, monitoring three ions per compound (Table 1). An MRM method was developed with the same instrument, keeping the temperature of the two quadrupoles at 150 ºC. Two transitions were monitored for each analyte, the first for quantification purposes and the second for confirmation. Table 1 shows the mass spectrometer conditions selected. Resolution was adjusted to 1.0 Da for quadrupole 1 and 3. The solvent delay was 5 min.

Table 1: Retention time, ions monitored in SIM mode, and selected transitions in the MS/MS method for the target compounds.

Compund	R.T.	GC-MS(SIM)	GC-MS/MS		
	(min)	Monitored ions *(m/z)* [a]	Parent ions *(m/z)*	Daughter ions *(m/z)* [a]	C.E. (eV) [a]
HMF	5.7	183, 128 [b], 109, 97 [b]	<u>183</u>/109	<u>111</u>/81	<u>20</u>/10
Patulin	6.9	226, 183, 154 [b], 136 [b]	<u>183</u>/226	<u>152</u>/170	<u>20</u>/10

[a] Underlined values were used for quantification.

[b] Ions corresponding to the non-derivatized species.

3. Results and discussion

3.1. Gas chromatography and MS/MS optimization

The separation of the two analytes of interest was achieved rapidly with a non-polar capillary column. Derivatized HMF appeared earlier in the chromatogram due to its lower boiling point. The molecular ion (*m/z* 198) was very small, as previously reported for cyclic alcohols [31], and thus its use as parent ion was unfeasible. On the basis of the fragmentations selected, 183→111 was used for quantification in HMF, resulting in the parent ion from the loss of methyl radical from the molecular ion $[M\text{-}CH_3]^+$. The daughter corresponded to the loss of the ODMS moiety followed by an opening of the furan ring. The transition selected for confirmation was 109→81, corresponding to a loss of OTMS to give the parent ion and a loss of COH to furnish the daughter ion of *m/z* 81 $[M\text{-}(OTMS+COH)]^+$. Concerning patulin, quantification transition 183→152, corresponding to the base peak $[M\text{-}(CH_3+CO)]^+$ followed by a loss of OCH_3, was selected. The transition used for confirmation was 226→170 from to consecutive losses of CO from the molecular ion (*m/z* 226). A reaction scheme of the fragmentations obtained can be found in Supplementary Material.

3.2. Extraction

EtOAc is a suitable extraction solvent for patulin and HMF in juice matrices [32]. Although other solvents such as acetonitrile [12, 17] and long-chain alcohols [13] could be used, acetonitrile requires a QuEChERS procedure to achieve phase separation. This procedure is followed by a cleanup step due to the presence in the extract of many sugars present in the sample. In addition, acetonitrile is not highly suited for GC because of its high expansion volume in the gas phase, which can increase the risk of exceeding the injection liner capacity since sample is injected together with the derivatization reagent in the injection-port derivatization approach. On the other hand, long-chain alcohols contain hydroxyl groups that would interfere in the injection-port derivatization, which is conducted without solvent evaporation, as previously stated.

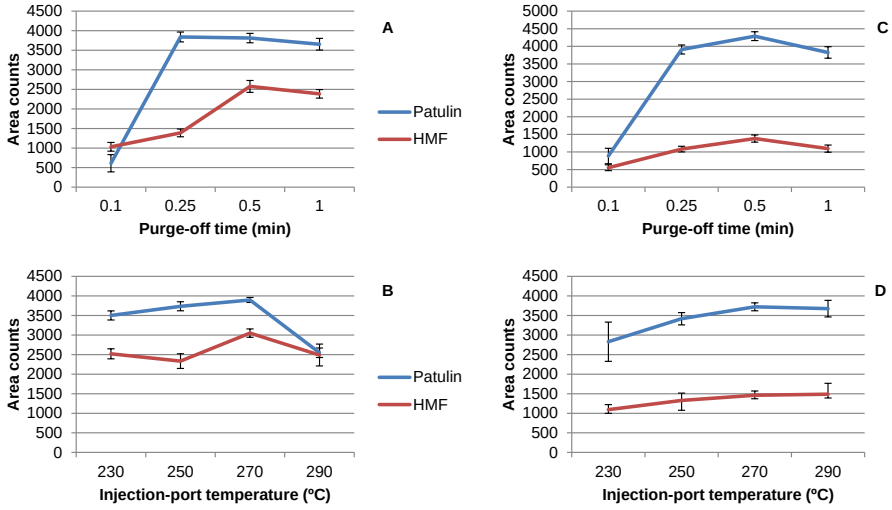

Figure 1: Optimization of injection-port conditions in cloudy apple juice (A and B) and concentrate apple juice (C and D). Error bars mean standard deviation (n=3).

3.3. Optimization of injection-port derivatization

Initially, injection mode was studied by using splitless, split, and pulsed pressure split/splitless mode to optimize the derivatization process performed in the injection port. Finally, the splitless mode was selected to perform further optimization of the methodology. Moreover, the injection-port silylation conditions were optimized in terms of time (purge-off), temperature, and sample volume/MSTFA ratio [26, 29] for both

compounds. Two apple derivatives were used, namely a sample of cloudy juice (Figure 1a and 1b) and a sample of concentrate (Figure 1c and 1d). Samples had an original HMF concentration and were spiked to obtain a concentration of 50 ng/mL of patulin. Both matrices showed a similar behavior. With respect to purge-off time, a high increase in the area counts was observed from 0.1 to 0.25 min. The area counts values were very similar from 0.25 to 0.5 min and started to decrease at 1 min. On the other hand, peak areas decreased at an injection-port temperature of 290 ºC in cloudy juices, although this decrease was found to be less significant for concentrate. The highest area for the two compounds was achieved using a 0.5 min purge-off time and 270 ºC injection-port temperature. The sample:derivatization reagent ratio was also optimized by studying ratios of 1:1, 1:2, 2:1 and 1:3 (v/v). The highest area was achieved using a sample: derivatization reagent ratio of 1:2. Figure 2 shows the chromatograms obtained in SIM mode monitoring the characteristic ions of the non-derivatized (Figure 2a) and derivatized (Figure 2b) compounds under study. Results showed complete derivatization of HMF and patulin as no peaks of the non-derivatized species were observed in the chromatogram of the derivatized compounds. Moreover, these figures show the increase on the chromatographic response of both compounds after derivatization.

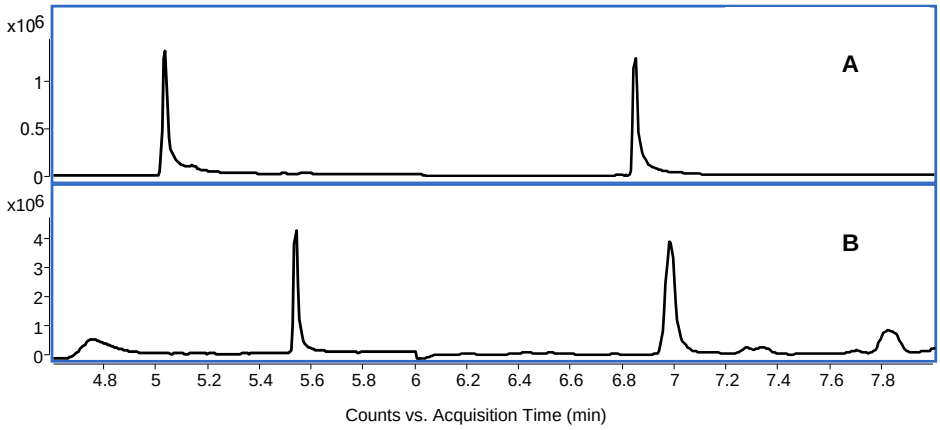

Figure 2: SIM chromatograms of ethyl acetate extracts spiked at 25 µg/mL for the non-derivatized (A) and 2.5 µg/mL for the derivatized sample (B). (Retention times for HMF: 5.05 and 5.55 for the non-derivatized and derivatized compound, respectively. Retention times for patulin: 6.90 and 6.98 for the non-derivatized and derivatized compound, respectively.)

3.4. Method performance

The performance parameters of the GC-MS/MS method for the optimized conditions described in section 3.1 were assessed in terms of LOD, LOQ, and intra- and inter-day repeatability (expressed as relative standard deviation), coefficient of determination (r^2), and linear range, as summarized in Table 2. A freshly prepared spiked apple juice not initially containing HMF or patulin was used to determine the LOD and LOQ. These two parameters were calculated as the analyte concentration, giving S/N= 3 and S/N=10, respectively. The results showed adequate performance in terms of LOD and LOQ, which are particularly critical for patulin in order to assure compliance with the strict regulations of the European Commission (10 μg/kg for food products destined for infants). The performance of the developed method for patulin was in the same range as other GC methods reported for the analysis of fruit juices (LOD=0.4 and LOQ=1.6 μg/kg) [24] and HPLC with DAD (LOD=0.4 and LOQ=2 μg/kg) [12] or MS (LOD=0.5 and LOQ=10 μg/kg) [17]. The analysis of HMF also offered a similar performance when compared with existing methods with LOD=6 and LOQ=20 μg/kg [9] and LOD=6.52 and LOQ=19.75 μg/kg when using SPME for liquid samples [25]. Regarding repeatability, the values were within those commonly accepted, whereas linearity proved excellent, with r^2 >0.991.

Table 2: Performance parameters of the GC-MS/MS method in terms of LOD, LOQ, intra- and inter-day repeatability (expressed as relative standard deviation), coefficient of determination (r2), and recoveries.

Compound	LOD (µg/kg)	LOQ (µg/kg)	Repeatability intra-day (%RSD, n=5)	Repeatability inter-day (%RSD, n=10)	r^2	linear range (µg/kg)	Recoveries lower level	Recoveries higher level	Ref.[1]
HMF	1.6	5	2.3-4.6	6.2-8.4	0.991	25-1000	74 ± 7	82 ± 6	[9, 25]
Patulin	0.7	2	4.6-5.7	8.3-11.7	0.999	5-192	87 ± 7	97 ± 4	[12, 17, 24]

[1] References of other methods that analyse the same compounds in similar samples.

Table 2 also shows the recoveries assessed by spiking a blank sample of freshly prepared apple juice at 5 and 50 µg/kg for patulin and 50 and 500 µg/kg for HMF (%RSD, n=5 is expressed in brackets). Recoveries of 82 (±7)% and 97 (±4)% for patulin and 76 (±7)% and 83 (±5)% for HMF were achieved. These results are in agreement with others reported in the literature for the liquid-liquid extraction of patulin [33, 34] and HMF [10] in fruit juices and prove the suitability of the methodology developed for the simultaneous analysis of patulin and HMF in these matrices.

3.5. Matrix effects

In an in-port derivatization method, matrix effects may occur in the injection liner, where the reaction takes place, due to the presence of co-extractives. For this reason, matrix effects were assessed by comparing the slopes attained with the standard additions method applied to two juice samples (concentrate and cloudy apple juice) and to standard dilutions. Six calibration concentrations were analyzed using the optimized experimental conditions (Table 3). Slopes were not found to be significantly different by a Students t-test ($\alpha < 0.05$). Hence, an external calibration curve was further applied to carry out the sample analyses.

Table 3: Slopes (area counts×mL/ng) achieved with the standard addition calibration curve.

Compound	Standard	Concentrate	Cloudy
HMF	35.7 ± 2.2	37.4 ± 1.3	42.9 ± 3.9
Patulin	55.6 ± 2.6	56.2 ± 4.3	55.6 ± 3.2

3.6. Application to the analysis of real samples

The method described herein was applied to the analysis of samples of commercial fruit juice, including several samples of juice concentrate. These concentrates initially ranged from 66 to 73 ºBrix and were diluted to 12 ºBrix prior to the analysis. HMF was present in all samples, with a concentration ranging from 0.14 to 3.05 µg/g (Table 4). In contrast, only two samples of concentrate quantifiable values of patulin, registering 4.54 and 18.91 µg/kg, respectively. The concentration of patulin in concentrate 2 was above the maximum content allowed for infant consumption (10 µg/kg). In addition, patulin was detected in two pear samples but in these cases it was below the LOQ. Finally, Figure 3 shows an MRM chromatogram resulting from the application of the described methodology to a juice sample.

Table 4: HMF and patulin concentration in fruit juice products.

Sample	HMF (µg/g)	Patulin (µg/kg)
	Mean ± RSD	Mean ± RSD
Apple concentrate 1	2.86 ± 0.24	4.54 ± 0.24
Apple concentrate 2	1.99 ± 0.07	18.91 ± 0.07
Apple concentrate 3	0.57 ± 0.15	<LOQ
Apple concentrate 4	3.05 ± 0.23	n.d.
Pear concentrate1	0.09 ± 0.001	<LOQ
Pear concentrate 2	0.86 ± 0.02	<LOQ
Pear concentrate 3	1.04 ± 0.28	n.d.
Pear concentrate 4	0.64 ± 0.04	n.d
Pear concentrate 5	0.61 ± 0.01	n.d.
Cloudy apple juice 1	0.48 ± 0.05	n.d
Cloudy apple juice 2	0.36 ± 0.02	n.d.
Cloudy pear juice 1	0.14 ± 0.01	n.d.
Cloudy pear juice 2	0.20 ± 0.01	n.d.

n.d.: not detected; <LOQ: detected but with a S/N<10.

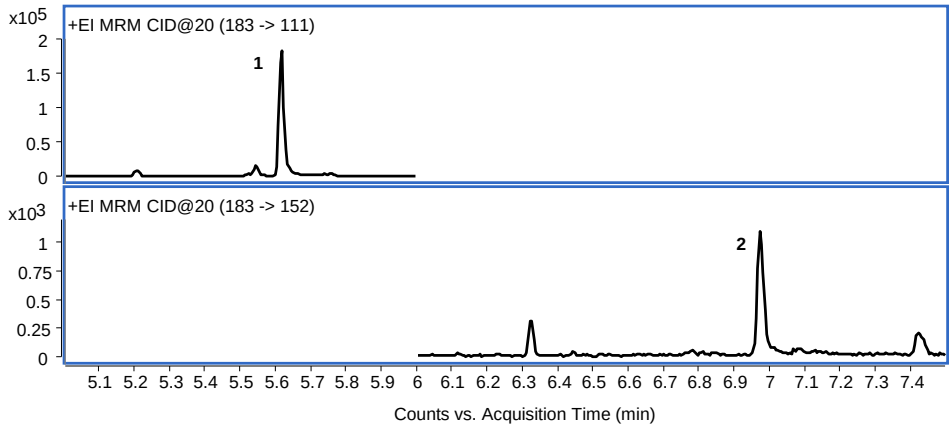

Figure 3: MRM chromatogram obtained for a derivatized apple juice sample with the quantification transition for: (1) HMF and (2) patulin.

4. Conclusions

The proposed analytical method, consisting of an injection-port derivatization coupled to gas chromatography tandem mass spectrometry, offers a novel approach for the simultaneous analysis of two key compounds, HMF and patulin, in the fruit juice industry. Samples can be analyzed avoiding concentration and purification processes. The chromatographic separation of the two compounds was achieved in a short chromatographic run (12.2 min) suitable for routine controls of these compounds in the fruit juice industry. The optimal conditions for the injection-port derivatization represent an important saving in terms of derivatization reagent consumption and sample preparation time. With the proposed method LOD and LOQ are below the maximum patulin concentration permitted for food products for infants and young children, thus this methodology is compliant with current legal standards. Finally to test the usefulness of the method, it was applied to various apple and pear juice samples from different origin. All of them contained HMF. In contrast, patulin was detected only in a few samples.

Acknowledgements

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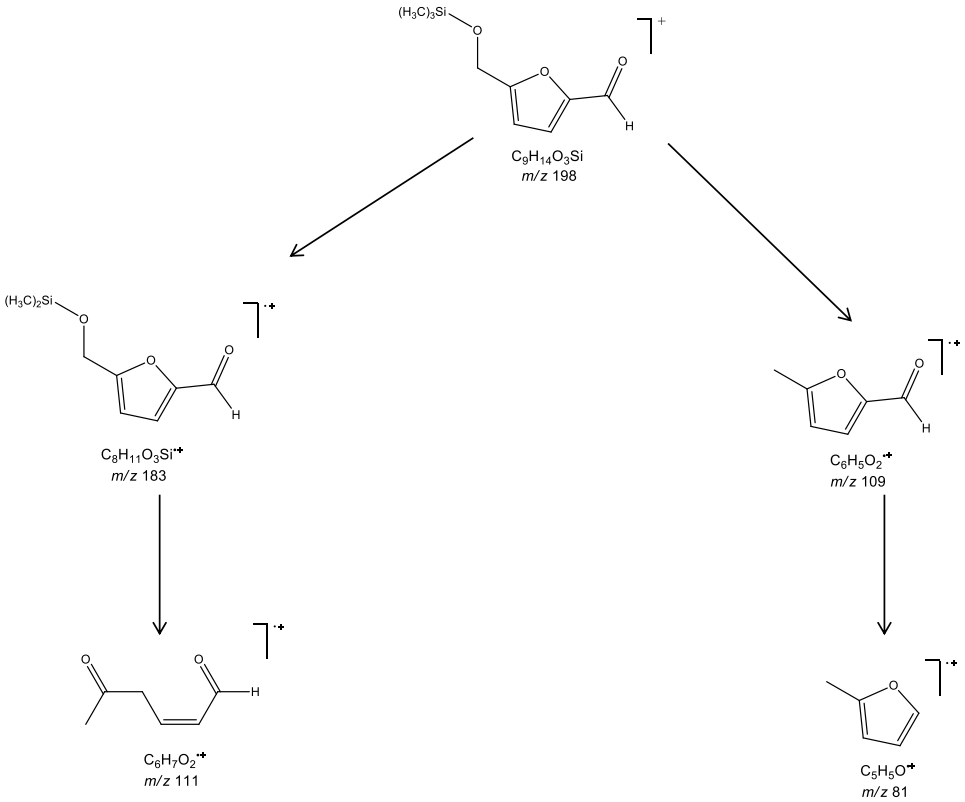
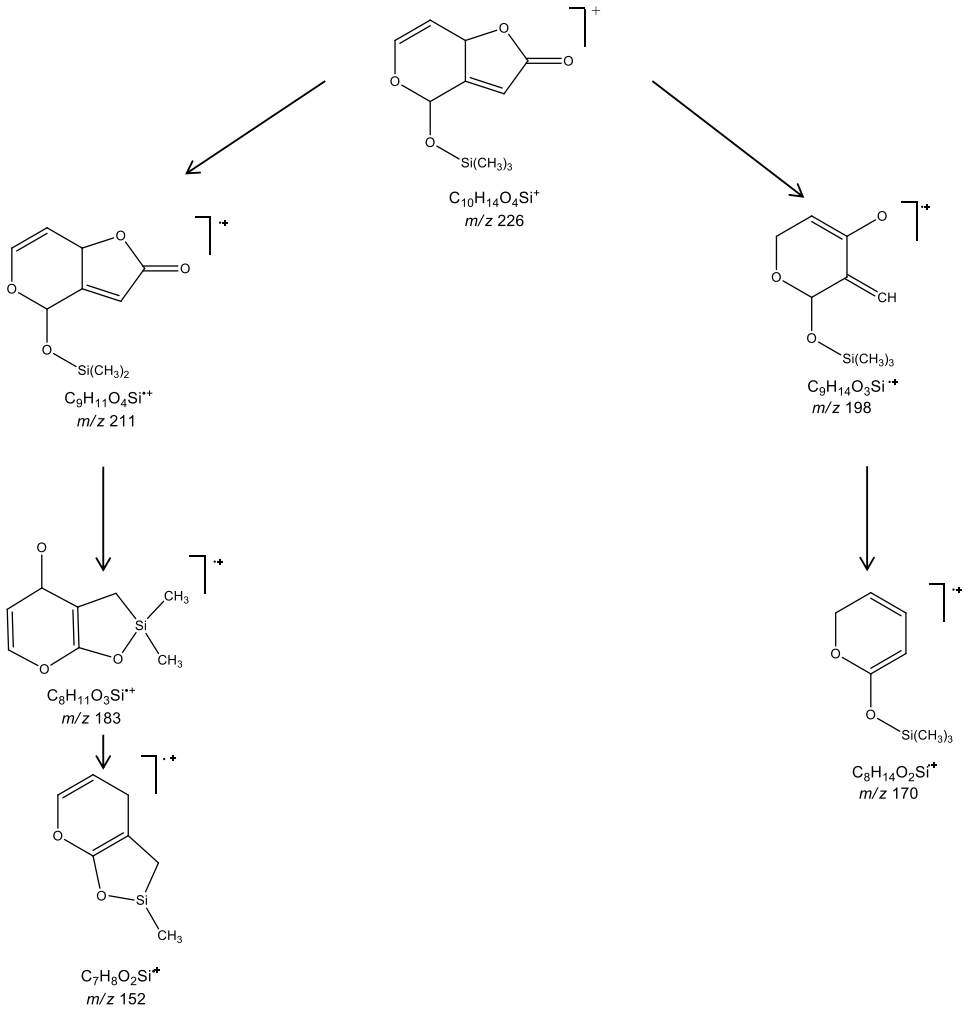
Supplementary data

SD1: Proposed fragmentation scheme for 5-hydroxymethylfurfural.



SD2: Proposed fragmentation scheme for patulin.



3.2.3. Dispersive liquid-liquid microextraction and injection-port derivatization for the determination of free lipophilic compounds in fruit juices by gas chromatography-mass spectrometry

DISPERSIVE LIQUID-LIQUID MICROEXTRACTION AND INJECTION-PORT DERIVATIZATION FOR THE DETERMINATION OF FREE LIPOPHILIC COMPOUNDS IN FRUIT JUICES BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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Abstract

A method consisting of dispersive liquid-liquid microextraction followed by injection-port derivatization and gas chromatography-mass spectrometry for the analysis of free lipophilic compounds in fruit juices is described. The method allows the analysis of several classes of lipophilic compounds, such as fatty acids, fatty alcohols, phytosterols and triterpenes. The chromatographic separation of the compounds was achieved in a chromatographic run of 25.5 min. The best conditions for the dispersive liquid-liquid microextraction were 100 µL of $CHCl_3$ in 1 mL of acetone. For the injection-port derivatization, the best conditions were at 280 ºC, 1 min purge-off, and a 1:1 sample:derivatization reagent ratio (v/v) using MSTFA:pyridine (1:1) as reagent. Quality parameters were assessed for the target compounds, giving a LOD ranging from 1.1 to 5.7 ng/mL and LOQ from 3.4 to 18.7 ng/mL for linoleic and stearic acid, respectively. Repeatability (%RSD, n=5) was below 11.51% in all cases. In addition, the method linearity presented an R^2 ≥0.990 for all ranges applied. Finally, the method was used to study the lipophilic fraction of various samples of commercial fruit juice.

Keywords: *DLLME, injection-port derivatization, GC-MS, free lipophilic fraction, fruit juices.*

1. Introduction

The lipophilic fraction of fruits and juices is considered to be influenced by cultivar, ripening, and season [1-3]. This fraction is composed by several classes of compounds, such as fatty acids, fatty alcohols, sterols and triterpenes. Fatty acids are primary metabolites, and in fruit samples they are present in saturated and/or unsaturated forms. Fatty alcohols are mainly long-chain primary alcohols, usually with an even number of carbon atoms. In most vegetable samples, they are present at minor concentrations [1, 4]. Phytosterols and triterpenes are secondary metabolites. The former are tretracyclic compounds similar to cholesterol. They occur in plants and vary only in carbon side chains and/or presence or absence of double bonds. These compounds have serum cholesterol-lowering effects [5, 6] and immune modulatory activity [7]. Given these properties, they have been widely studied in vegetables and fruits [8]. Triterpenes consist of a pentacyclic structure of six-isoprene units. Although their biological function and possible benefits for humans are still being addressed, anti-hypertensive, anti-atherosclerotic and anti-oxidant effects have already been reported [9].

Dispersive liquid-liquid microextraction (DLLME) was introduced 10 years ago by Assadi and coworkers [10]. In this method, which is based on ternary component solvent system, the appropriate mixture of extraction solvent and disperser solvent is injected into the aqueous sample, thereby producing a cloudy solution. After centrifugation, the organic layer is collected for analysis. This technique achieves high enrichment factors. In addition, speed and low consumption of organic solvents are two of the main advantages of this approach, which can be included in the group of clean chemistry procedures. Furthermore, the use of organic solvents in this technique makes it compatible with direct injection to a gas chromatograph. In contrast, the HPLC analysis of these compounds requires the evaporation of the organic solvent and reconstitution with an HPLC-compatible solvent.

Gas chromatography (GC) is generally the technique of choice when analyzing lipophilic compounds, such as fatty acids, fatty alcohols, phytosterols and triterpenes. In this case, the chromatograph is usually coupled to a flame ionization detector (FID) or to a mass spectrometry (MS) detector, the latter additionally providing spectra useful for identification purposes. However, as these compounds contain polar groups and in order to improve their performance in GC, derivatization is performed before subjecting samples to analysis. In this regard, fatty acids, which contain a carboxylic acid group, have been traditionally converted into their alkyl derivatives [11, 12], although conversion into silyl esters has also been widely used [13]. With respect to the other classes of compounds, fatty alcohols are generally silylated [1]. For sterols, acetylation [14] and silylation [15, 16] procedures have been described, while triterpenes are usually derivatized by silylation [17, 18].

Most of the derivatization protocols include off-line steps prior to the analysis. Off-line silylation procedures generally have the disadvantage of experimental errors, such as loss of analyte through evaporation and re-suspension steps, contamination of samples

during work-up, and interference of moisture in the reaction system, since silylating reagents and the resulting derivatives are extremely sensitive to the presence of water. On-line derivatization techniques have emerged in recent years [19-21]. These approaches allow a reduction of time-consuming sample processing steps, a decrease in the amount of reagents required, and an increase in the efficiency of the analysis. Inlet-based or in-port derivatization is one of these alternative approaches. This on-line process involves introducing the sample and derivatization reagent directly into the hot GC inlet, where the derivatization reaction takes place in the gas-phase [22]. The sample and the derivatization reagent can be injected separately. This can be achieved by first injecting the sample or the derivatization reagent manually [23], thus calling for the presence of an analyst. Alternatively, injection of the sample and reagent can be attained simultaneously by using a software-controlled sandwich injection. In this case, the syringe is filled with both the sample and the derivatization reagent, allowing an air gap between them. The latter approach is expected to give better results in terms of repeatability and automation of the analytical sequence.

Here we sought to develop a new method for the analysis of the free lipophilic fraction of several fruit juices. As we were dealing with liquid samples, a DLLME method was optimized by full factorial experimental design. Furthermore, derivatization was optimized in terms of derivatization reagent, injection-port temperature, purge-off time, and sample:derivatization reagent volume ratio. Furthermore, a microextraction technique was used, which reduces solvent consumption and circumvents the evaporation step prior to derivatization. Consequently, this novel method allows a reduction of analyte degradation and sample preparation time.

2. Material and methods

2.1. Reagents, solvents, standard solutions and samples

N-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and chlorotrimethylsilane (CTMS) were purchased from Sigma-Aldrich (Buchs, Switzerland), chloroform ($CHCl_3$) and pyridine from J.T. Baker (Deventer, The Netherlands), and chlorobenzene, methanol, and acetone from Sigma-Aldrich. Standards of sugars and organic acids to prepare the synthetic juice, namely glucose, fructose, sucrose, sorbitol and malic acid were purchased from Sigma-Aldrich [24].

Stock standard solutions of fatty acids (palmitic acid, linoleic acid, oleic acid and stearic acid), fatty alcohols (docosanol, tetracosanol, hexacosanol and octacosanol), sterols (campesterol, stigmasterol and β-sitosterol), and triterpenes (α-amyrin, oleanolic acid and ursolic acid) were prepared from the corresponding analytical standards (Sigma-Aldrich). Working solutions of 100 µg/mL and 10 µg/mL were prepared with acetone from consecutive dilutions of the stock solutions. All standard solutions were stored at -20 ºC and warmed to room temperature before use.

Berry, cloudy apple, apple concentrate, mango, pear, peach, orange (x2), a mixture of carrot and orange, and pineapple juice were purchased from a local supermarket.

2.2. Instrumentation

The GC-MS analyses were performed on an Agilent 7890 GC (Agilent Technologies, Palo Alto, CA, USA) with a multimode injector and a splitless liner containing a piece of glass wool. A fused silica high-temperature capillary column J&W DB–5MS (30 m × 0.25 mm i.d.; 0.25 µm film thickness) from Agilent was used at constant flow. The detector was an Agilent 7000B triple quadrupole mass spectrometer with inert EI ion source. The mass spectrometer worked in SIM mode with EI ionization source at 70 eV. Helium with a purity of 99.9999% was used as carrier and quenching gas, and nitrogen with a purity of 99.999% as collision gas, both supplied by Air Liquide (Madrid, Spain).

For control purposes and data analysis, Agilent Mass Hunter B.06.00 software was used.

2.3. Analytical procedure

Initially, juice samples were centrifuged for 5 min at 5000 rpm to eliminate solid particles. Subsequently, a 0.5-mL aliquot of juice was diluted to 5 mL with water in a 15-mL screw cap glass tube with a conical bottom. Then, 1 mL of acetone (dispersive solvent) containing 100 µL of chloroform (extractant solvent) was rapidly injected into the aqueous solution using a micropipette, and the mixture was vigorously hand-shaken for several seconds. A cloudy solution consisting of very fine droplets of chloroform dispersed through the sample solution was formed, and the analytes were extracted into the fine droplets. After centrifugation for 5 min at 3000 rpm, the extraction solvent was deposited at the bottom of the conical tube. The deposited phase was collected and transferred to a crimp-cap vial containing a glass insert for injection into the gas chromatograph.

Sandwich injections of the sample and the derivatization reagent MSTFA:pyridine (1:1, v/v) in a volume ratio of 1:1 were carried out in splitless mode at 280 ºC. The inlet insert was a silanized glass tube containing a piece of glass wool. The purge-off time was set at 1 min. The gas chromatograph temperature was programmed as follows: 150 ºC (held for 1 min) to 220 ºC at 20 ºC/min and to 320 ºC at 5 ºC/min (held for 1 min) at a constant flow regime of 2 mL/min. The cap of the vial containing the derivatization reagent was PTFE/Silicone/PTFE, which allows repeated injections. This cap was replaced every 20 injections to prevent contamination from the septum.

The temperatures of the transfer line, ion source, and quadrupole were 300 ºC 250 ºC and 150 ºC, respectively. The mass spectra detector operated in selected ion monitoring (SIM) mode, monitoring two or three ions per compound (Table 1), one for quantification and the others for confirmation purposes. Segmentation of the SIM method was performed to allow a higher scan time for each ion. The solvent delay was 4 min.

Table 1: Retention time and monitored ions in SIM mode for the target compounds.

Compound	Molecular weight (amu)[a]	R.T. (min)	Segment	Monitored ions *(m/z)* [b]
Palmitic acid	328	5.87	1	313, 328
Linoleic acid	352	7.14	2	337, 352
Oleic acid	354	7.18	2	339, 354
Stearic acid	356	7.39	2	341, 356
Docosanol	383	10.44	3	367, 383
Tetracosanol	411	12.71	3	395, 411
Hexacosanol	439	15.05	4	423, 439
Octacosanol	467	17.41	4	451, 467
Campesterol	472	18.78	5	343, 382, 472
Stigmasterol	484	19.13	5	394, 484
β-Sitosterol	486	19.77	6	357, 396, 486
α-Amyrin	498	20.26	6	218, 498
Oleanolic acid	601	22.33	7	320, 482
Ursolic acid	601	22.82	7	320, 482

[a] Molecular weights corresponding to the TMS-derivatized species.

[b] Underlined values were used for quantification.

3. Results and discussion

3.1. Optimization of injection-port derivatization

The first step involved the study of the injection mode: splitless, split, and pulsed pressure split/splitless modes were tested. Considering these preliminary results (results not shown), the splitless injection mode was selected for further optimization of the method. Moreover, for all compounds under analysis, the injection-port silylation conditions were optimized in terms of derivatization reagent, temperature, time (purge-off), and sample:derivatization reagent volume ratio. These are the parameters typically optimized in such a derivatization procedure [19, 23]. For this purpose, a standard mixture containing all the compounds at a concentration between 1.95 and 6.24 µg/mL was used.

The standard compounds were quantified using an external calibration curve containing the off-line derivatized analytes. Initially, several derivatization reagent combinations were tested: MSTFA, MSTFA: pyridine (1:1, v/v), BSTFA, BSTFA: pyridine (1:1, v/v), and BSTFA (1% CTMS). Figure 1 shows that the derivatization reagent had a low influence for fatty acids. However, a higher influence was observed for the other compounds under analysis, MSTA, MSTFA:pyridine and BSTFA:pyridine being those that showed the best performance. Of these, the silylating reagents that contained pyridine gave the best results, thereby confirming that pyridine catalyzes the reaction, as already described [25]. MSTFA:pyridine was finally selected from the two reagents containing pyridine. This derivatization reagent shows a superior performance in the case of ursolic acid, which is the compound that showed the lowest response under all the conditions tested. Next, temperature of the injection-port was varied from 200 ºC to 300 ºC in 20 ºC intervals to find the most appropriate conditions. Figure 2 shows that 200 ºC was too low in all cases. With respect to fatty acids and fatty alcohols, both classes presented a similar performance in a range from 220 ºC to 300 ºC. On the other hand, for sterols and triterpenes, the best results were achieved at 260 ºC and 280 ºC. For these reasons, an injection-port temperature of 280 ºC was selected. The next step consisted of assaying several purge-off times (0.1, 0.25, 0.5, 1 and 2 min) in splitless mode. Figure 3 shows that the best performance was obtained using 1- and 2-min purge-off times in all cases. Therefore, a 1-min purge-off time was finally selected to perform further sample analysis. Finally, several volume ratios between sample and derivatization reagent (1:1, 1:2 and 1:3) were tested. These ratios were selected paying attention not to exceed injection liner capacity. Figure 4 shows no influence of the sample:reagent ratio on the final results. Consequently, a 1:1 sample:derivatization reagent ratio (v:v) was selected.

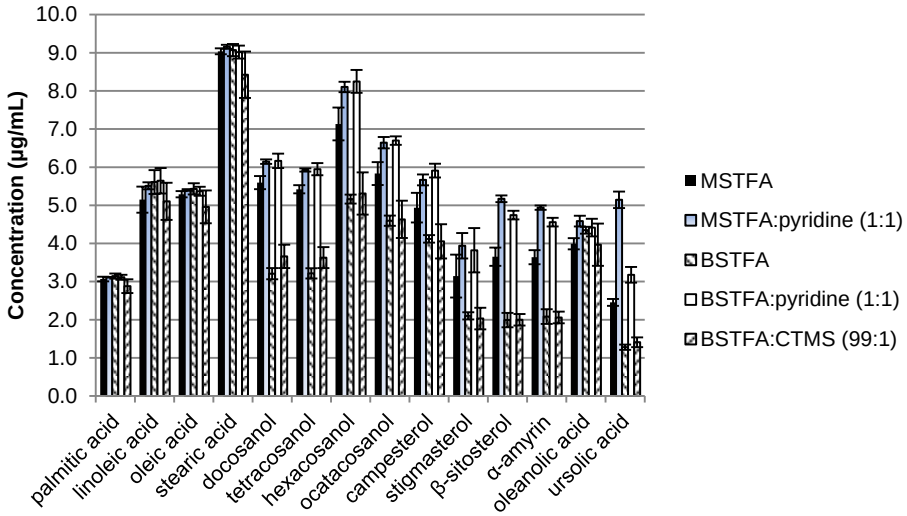

Figure 1: Optimization of in-port derivatization according to derivatization reagent. Vertical segments indicate standard deviation (n=3).

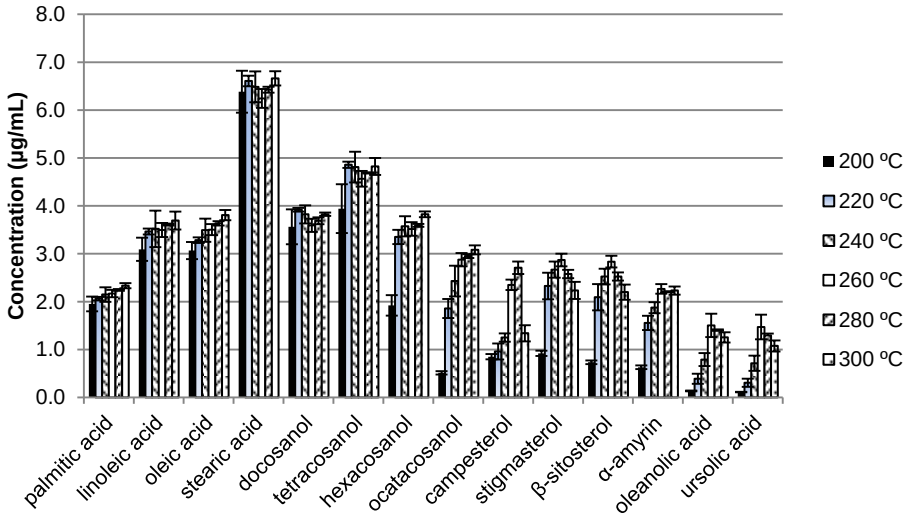

Figure 2: Optimization of in-port derivatization according to injection-port temperature. Vertical segments indicate standard deviation (n=3).

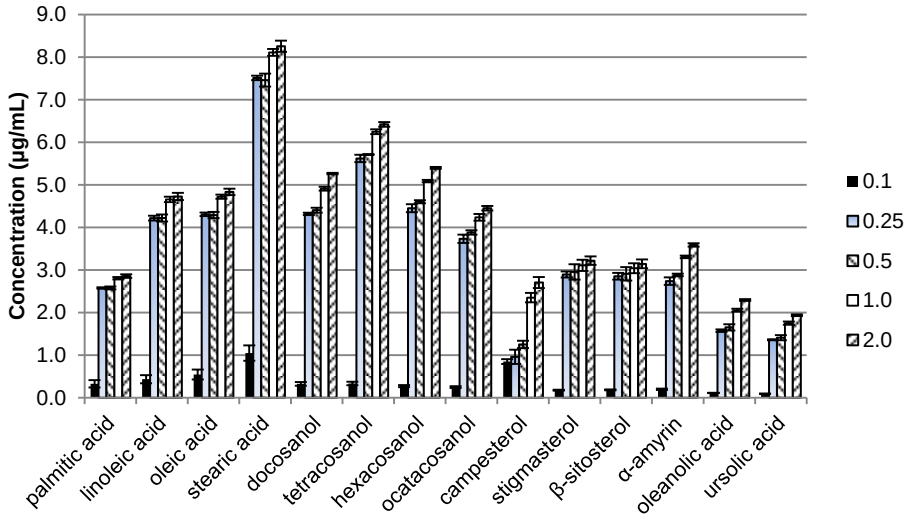

Figure 3: Optimization of in-port derivatization according to purge-off time. Vertical segments indicate standard deviation (n=3).

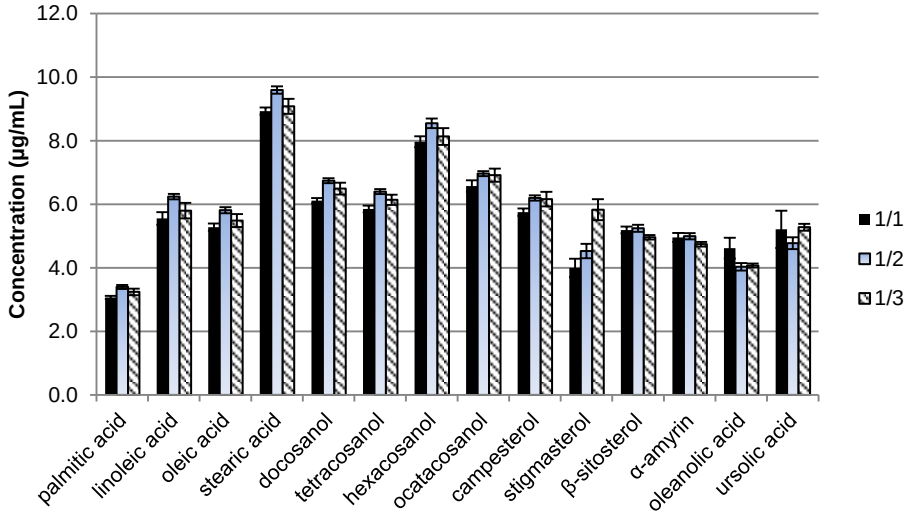

Figure 4: Optimization of in-port derivatization according to sample: derivatization reagent volume ratio. Vertical segments indicate standard deviation (n=3).

3.2. Dispersive liquid-liquid micro-extraction (DLLME) optimization

DLLME conditions were optimized by means of a full factorial experimental design using a synthetic juice spiked with all the compounds of interest at 20 ng/mL. The extracts were analyzed under the optimized in-port derivatization conditions described in section 3.1. The following three factors were studied: 1) Extraction solvent, which must meet the following four requirements: higher density than water; good chromatographic behavior; high extraction capacity for the compounds of interest, and low water solubility [26, 27]. Chloroform and chlorobenzene were the two solvents assayed; 2) Extraction solvent volume, which was assayed at 50, 100 and 200 µl; and 3) Dispersive solvent. These must be highly miscible with the aqueous sample and the extraction solvent. Moreover, they should decrease the interfacial tension of the extractant in order to make the droplet size small, thereby increasing the extraction efficiency [26]. Methanol and acetone were the two candidates that accomplished the required properties. For each experiment, the concentrations of the analytes were calculated using an external calibration curve. Enrichment factors (EFs), i.e. the ratio of the analyte concentration in the deposited phase to the initial concentration in the aqueous phase, were also calculated. To facilitate the interpretation of the results of the experimental design, an analysis of variance (ANOVA) was used to describe the effect of the studied factors on the EFs achieved. Table 2 shows the results of the p-values used to determine the statistical significance of each factor and their interactions during DLLME optimization. Extraction solvent volume was significant at 95% confidence level (p value <0.05) for all compounds; this is a logical effect as the lower the volume of the organic phase, the higher the concentration of the analytes. However, it is sometimes difficult to collect very low volumes and achieve an acceptable repeatability for all samples. Consequently, the selection of higher extraction volumes is recommended. Extraction solvent was another factor that was statistically significant. $CHCl_3$ gave higher EFs for several analytes of various classes, such as palmitic acid, stearic acid, campesterol, α-amyrin, oleanolic acid, and ursolic acid. Finally, the interaction between solvent and disperser was significant for oleanolic and ursolic acids. The other factor (disperser) and the other possible interactions were not significant. As can be seen in Figure 5, mean charts for the extraction solvent shows that higher EFs were attained for all compounds when using chloroform (Figure 5a, b, c, d, e, f), and the interaction of extraction solvent and disperser that showed the best combination was that formed by chloroform and methanol (Figure 5g, h).

Table 2: P-values achieved in the ANOVA for the synthetic juice spiked at 20 ng/mL in the full factorial experimental design[1].

Compound	A: Solvent	B: Extraction solvent volum	C: Disperse	AB	AC	BC
Palmitic acid	**0.038**	**0.008**	0.058	0.438	0.052	0.631
Linoleic acid	0.109	**0.017**	0.376	0.393	0.158	0.785
Oleic acid	0.126	**0.015**	0.743	0.490	0.112	0.561
Stearic acid	**0.025**	**0.008**	0.058	0.260	0.053	0.516
Docosanol	0.083	**0.041**	0.184	0.245	0.117	0.948
Tetracosanol	0.081	**0.042**	0.218	0.247	0.128	0.967
Hexacosanol	0.082	**0.045**	0.239	0.259	0.145	0.965
Octacosanol	0.091	**0.046**	0.295	0.264	0.158	0.968
Campesterol	**0.029**	**0.045**	0.209	0.224	0.088	0.912
Stigmasterol	0.083	**0.042**	0.291	0.241	0.125	1.000
β-Sitosterol	0.079	**0.038**	0.329	0.233	0.111	0.981
α-Amyrin	**0.035**	**0.032**	0.161	0.152	0.092	0.920
Oleanolic acid	**0.039**	**0.017**	0.097	0.125	**0.036**	0.545
Ursolic acid	**0.047**	**0.015**	0.097	0.134	**0.036**	0.573

[1] Values in bold indicate statistical significance (p-value <0.05).

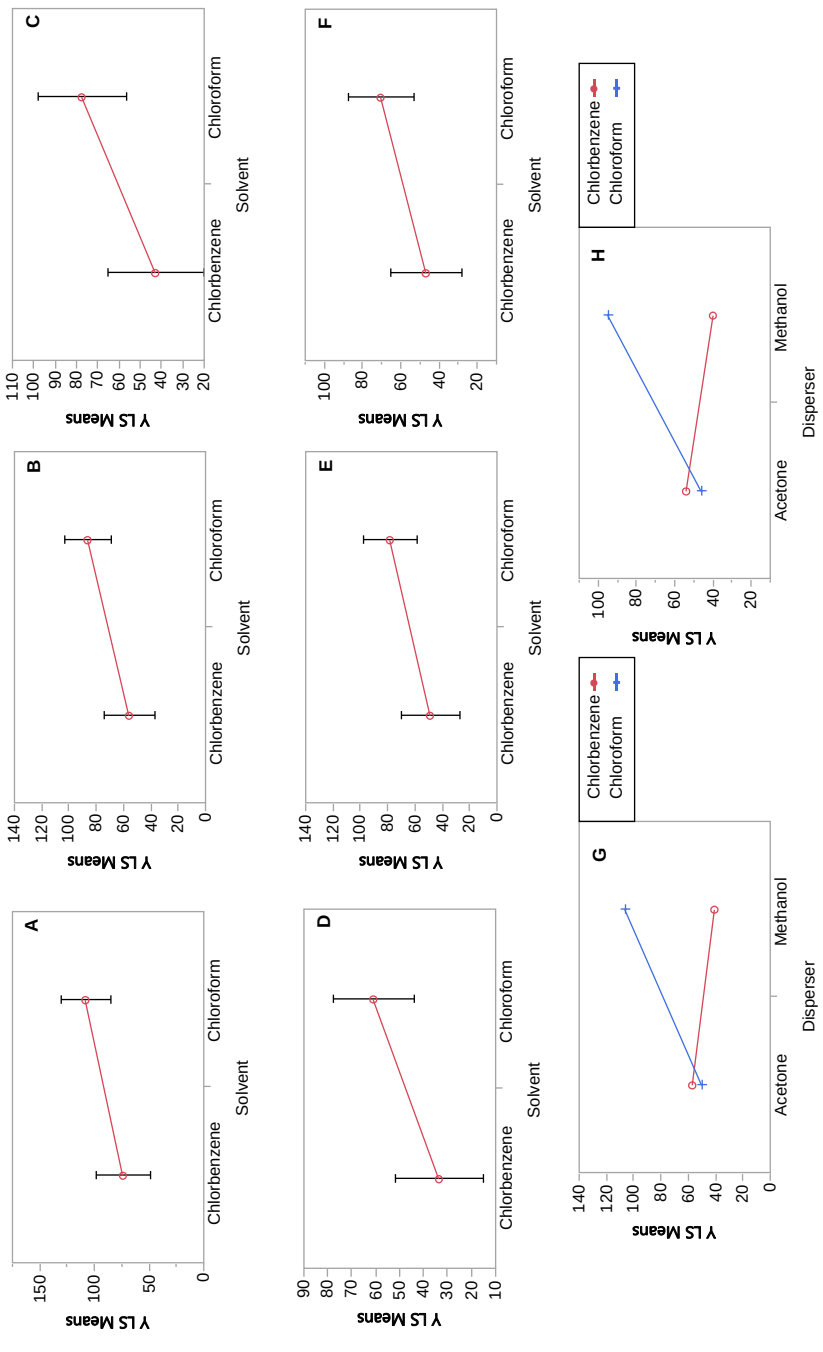

Figure 5: Mean charts for the extraction solvent: a) palmitic acid, b) stearic acid, c) campesterol, d) α-amyrin, e) oleanolic acid, f) ursolic acidand for the interaction of extraction solvent and disperser: g) oleanolic acid and h) ursolic acid.

3.3. Method performance

The performance parameters of the GC-MS method for the optimized conditions were assessed in terms of limits of detection (LOD), limits of quantification (LOQ), coefficient of determination (r^2), linear range, and repeatability, as summarized in Table 3. A synthetic juice was used for this purpose. LOD and LOQ were calculated as the concentration giving a signal to noise ratio of three (S/N=3) and ten (S/N=10), respectively. In this regard, the LOD obtained were low, ranging from 1.1 to 5.7 ng/mL while the LOQ ranged from 3.4 to 18.7 ng/mL. Considering linearity, calibration standards were prepared by spiking the synthetic juice. In all cases, good linearity was achieved for all compounds, with r^2 values of between 0.990 and 0.999. However, broader linear ranges, from 10 to 1050 ng/mL and from 10 to 1500 ng/mL, were achieved for unsaturated fatty acids (linoleic and oleic acid) and β-sitosterol and oleanolic acid, respectively. In contrast, saturated fatty acids (palmitic and stearic acid) and fatty alcohols presented a narrower linear range compared to the other compounds. Moreover, repeatability (expressed as %RSD, n=5) achieved values below 11.51%.

Table 3: Performance parameters of the DLLME-GC-MS method in terms of LOD, LOQ, coefficient of determination (r^2), linear range, and repeatability.

Compound	**LOD (ng/mL)**	**LOQ (ng/mL)**	**r^2**	**linear range (ng/mL)**	**repeatability (RSD, %)**
Palmitic acid	3.1	10.0	0.990	10-120	6.92
Linoleic acid	1.1	3.4	0.999	10-1050	3.63
Oleic acid	1.2	3.6	0.998	10-1050	5.72
Stearic acid	5.7	18.7	0.991	20-400	9.00
Docosanol	3.1	10.2	0.997	10-500	8.28
Tetracosanol	3.2	10.4	0.990	10-200	10.52
Hexacosanol	1.5	4.6	0.997	10-500	11.51
Octacosanol	1.6	4.8	0.995	10-500	7.65
Campesterol	2.2	5.8	0.993	6-500	7.63
Stigmasterol	3.3	9.8	0.990	10-200	9.13
β-Sitosterol	3.1	9.9	0.996	10-1500	4.54
α-Amyrin	3.6	9.8	0.996	10-500	11.09
Oleanolic acid	3.9	9.5	0.997	10-1500	7.95
Ursolic acid	3.8	10.0	0.993	10-200	6.00

3.4. Recovery assays

The analytical procedures described in sections 3.1 and 3.2. were tested in commercial juice samples in order to check the performance of the method in this matrix. Before proceeding, samples were centrifuged and diluted 1:10 with distilled water to eliminate solid particles and minimize possible matrix effects, as the effectiveness of a DLLME procedure is highly related to aqueous-like matrices.

Recovery assays were carried out in two samples (berry and peach juice) and analyzed using DLLME and injection-port derivatization. To perform recovery experiments, samples were spiked at 40 ng/mL with all analytes and recoveries were calculated with the following equation (1):

$$\%R = (C_{found} - C_{real}) \div C_{added} \times 100 \qquad (1)$$

We observed that an interphase formed when using methanol as disperser. This effect led to a reduction of the organic phase volume, thus precluding good and repeatable recovery values. On the other hand, when acetone was used as disperser, recoveries were acceptable for all compounds (Table 4), although we reported lower values for fatty alcohols (57-78% depending on the compound). Recoveries were close to 100% for fatty acids and ranged from 61 to 85% for sterols and triterpenes in berry juice. On the other hand, for peach juice, the recoveries for most sterols and triterpenes showed values close to 100%. For the abovementioned reasons, acetone was chosen as disperser and with a volume of 100 µL of $CHCl_3$ as the best conditions to perform the lipophilic fraction analysis of the juice samples.

Table 4: Recoveries (%) and RSD (%, n=3) for berry and peach juice samp
spiked at 40 ng/mL with all the compounds under study.

Compound	Berries juice	Peach juice
Palmitic acid	95 (±4)	96 (±7)
Linoleic acid	98 (±6)	92 (±8)
Oleic acid	92 (±7)	89 (±5)
Stearic acid	95 (±5)	99 (±4)
Docosanol	64 (±8)	57 (±5)
Tetracosanol	60 (±2)	78 (±4)
Hexacosanol	59 (±3)	65 (±2)
Octacosanol	57 (±6)	64 (±6)
Campesterol	78 (±4)	102 (±4)
Stigmasterol	79 (±5)	104 (±8)
β-Sitosterol	84 (±3)	109 (±3)
α-Amyrin	61 (±5)	65 (±7)
Oleanolic acid	85 (±2)	92 (±4)
Ursolic acid	78 (±3)	91 (±3)

3.5. Application to the analysis of commercial samples

Finally, the method described herein was applied to the analysis of ten samples of commercial fruit juice, including berry, apple, mango, pear, peach, orange, apple and carrot, pineapple, and apple concentrate, with the aim to assess their lipophilic composition. Samples were analyzed in triplicate. Figure 6 shows a chromatogram corresponding to a sample of apple juice attained with the proposed methodology.

Table 5 shows the concentration of the compounds under study. Palmitic acid was found in a homogenous range of 436-976.8 ng/mL in all samples, except apple concentrate, which presented the lowest concentrations of all compounds. This observation is explained by the fact that this concentrate contained a very low percentage of fruit. The behavior of linoleic acid content differed, varying from 620.5 ng/mL in mango to 4300.0 ng/mL in orange juice. Oleic acid was found at a range from 221.0 ng/mL in peach to 889.2 ng/mL in mango, while stearic acid—the fatty acid that was generally found at a lower concentration in our study—ranged from 70.0 ng/mL in pineapple to 369.1 ng/mL in apple juice. The results for fatty acids are consistent with those previously reported in apple, where linoleic acid was the most abundant followed by oleic and palmitic acid and a lower content of stearic acid [28], and in pear, where palmitic acid content was higher than oleic acid [29]. Fatty alcohols presented concentrations below the LOQ in orange, pineapple, apple and carrot juice, and apple concentrate juice. However,

the other samples presented measurable amounts of the four fatty alcohols under analysis. In this regard, docosanol ranged from 8.9 ng/mL in mango to 215.9 ng/mL in pear juice. On the other hand, for the other alcohols, i.e. tetracosanol, hexacosanol and octacosanol, the highest concentrations were found in apple juice, while berry and mango juice presented the lowest concentrations in all cases. The observation that apple juice had the highest content of hexacosanol and octacosanol is consistent with previous reports [30]. Regarding phytosterols, campesterol ranged from 30.5 ng/mL in apple concentrate to 1,423.3 ng/mL in orange juices, stigmasterol presented the highest concentration in peach juice (4,298.7 ng/mL), and β-sitosterol ranged from 305.2 ng/mL in berry juice to 4,298.7 ng/mL in apple juice. The results for sterols are similar to those previously reported. β-Sitosterol was the main sterol in orange [31], pineapple, mango, and apple juice, followed by campesterol [32]. Finally, triterpenes were found in only some of the samples, namely apple, pear, and peach, at a range from 205.0 to 848.9 ng/mL. These results are in agreement with previously ones reporting the presence of oleanolic and ursolic acids in apple skin [33] and pear fruit [34].

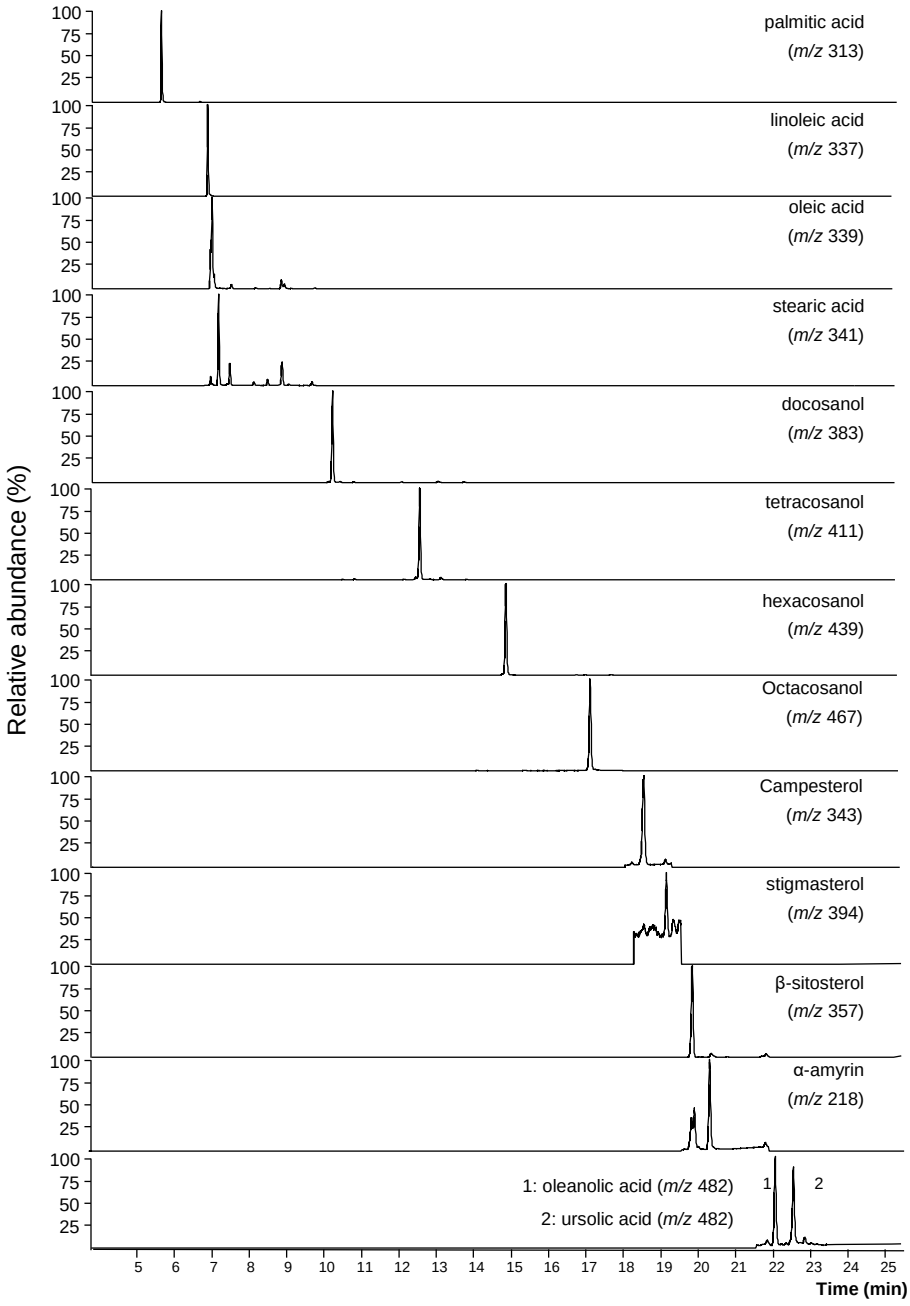

Figure 6: SIM chromatogram of an apple juice sample obtained with the proposed methodology.

Table 5: DLLME-GC-MS analysis of commercial fruit juice samples (ng/mL).

Compounds	Apple juice	Apple conc. juice	Pear juice	Mango juice	Peach juice	Orange juice	Orange juice 2	Apple and carrot juice	Pineapple juice	Berries juice
Palmitic acid	865	n.d.	754.9	956	743.6	837.2	976.8	819.2	673.9	436
Linoleic acid	4087.9	11.4	3142	620.5	2477.4	3098.5	4300	4244.7	1123.6	1350.1
Oleic acid	609.6	n.d.	573.7	889.2	221	616.3	849.6	569.1	876.2	669.1
Stearic acid	369.1	n.d.	282.6	116.7	173.5	87.4	168.1	139.5	70	86.2
Docosanol	21.4	<LOQ	215.9	8.9	13.9	<LOQ	<LOQ	<LOQ	<LOQ	11.6
Tetracosanol	262.8	<LOQ	145.6	78.2	73.2	<LOQ	<LOQ	<LOQ	<LOQ	5.4
Hexacosanol	545.2	11.7	91.9	33.7	103.7	<LOQ	<LOQ	<LOQ	<LOQ	18.8
Octacosanol	727.1	18.1	106.9	32.1	85.5	<LOQ	20.7	23.6	28.1	33.4
Campesterol	584.1	30.5	293	624.8	485.1	1279.9	1423.3	1183.5	1151.9	166.5
Stigmasterol	105.8	10.2	18.5	150.1	1801.6	414.3	383.3	811.8	87.5	33.1
β-Sitosterol	4298.7	n.d.	2739.6	1304.9	2101	3464.2	3322	2714.5	2012.4	305.2
α-Amyrin	98.5	n.d.	<LOQ	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.
Oleanolic acid	267.7	n.d.	<LOQ	n.d.	205	n.d.	<LOQ	n.d.	n.d.	n.d.
Ursolic acid	848.9	<LOQ	403.2	<LOQ	261.5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

n.d.: not detected; <LOQ: detected but with an S/N <10.

4. Conclusions

The analysis of free lipophilic compounds in liquid matrices can be effectively achieved using DLLME with $CHCl_3$ and acetone as extraction and dispersion solvents, respectively. DLLME allows a reduction in solvent volume. In addition, injection-port derivatization is a useful on-line technique to analyze compounds containing hydroxyl groups that could compromise their volatility and hence suitability for direct analysis by GC. Indeed, when using MSTFA:pyridine (1:1, v/v) as silylation reagent at optimized injection-port conditions, the method showed satisfactory analytical performance in terms of LOD, linearity (r^2 >0.990) and repeatability (RSD <11.51%). Moreover, with this analytical procedure, micro-volumes of reagents are used both in the extraction and derivatization stages, thus greatly reducing the amounts of reagents required. Finally, the analysis of commercial samples of fruit juice revealed the usefulness of the proposed methodology. The results achieved are in agreement with those reported in previous studies. Hence, in addition to fruit juice, this methodology could find application in other procedures aimed to analyze the free lipophilic fraction of aqueous matrices.

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3.3. Analysis of volatile and semi-volatile compounds in fruit-derived samples employing solid-phase microextraction techniques

The last Chapter is focused on the analysis of another group of compounds, as mentioned in the Introduction, composed by volatile and semivolatiles. In regards of the extraction method other techniques can be applied in contrast high boiling compounds. The fact that we are dealing with volatile compounds allows the use of thermal desorption combined with solid-phase microextraction resulting in the abolishment of organic solvents during sample preparation.

The first section of this Chapter reports the analysis of volatile compounds and enantiomeric analysis of chiral terpenoids in nine fruit and vegetable fibres resulting from juice industry by-products. Unlike physicochemical properties [1], volatile composition of fruit fibres has been very little studied. Only the volatile fraction of apple pomace has been previously addressed [2]. This part of the Thesis was carried out during a 3-month stay at the laboratory of Phytochemical Analysis of the Università degli Studi di Torino (Italy) under the guidance of Prof. Dr. Carlo Bicchi.

The second section of this Chapter consists on the development and application of stir bar sorptive extraction with a PDMS-coated stir bar [3] followed by thermal desorption and GC-MS for the analysis in direct immersion mode of the volatile and semi-volatile compounds present in peach juice samples. With the aid of common chemometric techniques such as PCA and SLDA, we show that it exist compositional differences that allow to distinguish between peach varieties and their processing conditions.

The results of this Chapter have been submitted for publication in *Journal of the Science of Food and Agriculture* and *Journal of Agricultural and Food Chemistry.*

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3.3.1. Characterization of the volatile composition and enantioselective analysis of chiral terpenoids of nine fruit and vegetable fibres resulting from juice industry by-products

CHARACTERIZATION OF THE VOLATILE COMPOSITION AND ENANTIOSELECTIVE ANALYSIS OF CHIRAL TERPENOIDS OF NINE FRUIT AND VEGETABLE FIBRES RESULTING FROM JUICE INDUSTRY BY-PRODUCTS

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Abstract

Fruit and vegetable fibres resulting as by-products of the fruit juice industry have won popularity because they can be valorised as food ingredients. In this regard bioactive compounds have already been studied but little attention has been paid to their remaining volatiles. Considering all the samples 57 volatiles were identified. Composition greatly differed between citrus and non-citrus fibres. The former presented over 90% of terpenoids, limonene being the most abundant and ranging from 52.7% in lemon to 94.0% in tangerine flesh. Non-citrus fibres showed more variable compositions, the predominant classes being aldehydes in apple (57.5%) and peach (69.7%), esters (54.0%) in pear, and terpenoids (35.3%) in carrot fibres. In addition, enantioselective analysis of some of the chiral terpenoids present in the fibre revealed that the enantiomeric ratio for selected compounds was similar to the corresponding volatile composition of raw fruits and vegetables and some derivatives, with the exception of terpinen-4-ol and α-terpineol, which showed variation, probably due to the drying process. The processing to which fruit residues to obtain fruit fibres showed to produce fibres with low volatile content for non-citrus products. Otherwise, citrus fibres analysed still presented a high volatile composition when compared with non-citrus.

Keywords: *HS-SPME-GC-MS, enantioselective analysis, volatile composition, fruit fibres, industrial process.*

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1. Introduction

The recovery, recycling, and upgrading of waste material is particularly relevant in the food and food processing industry, in which waste, effluents, residues, and by-products can be reclaimed and often turned into useful higher value-added products [1]. The food industry can take advantage of the physicochemical properties of these products to improve the viscosity, texture, sensory characteristics and shelf-life of final products. Hence, fibre-rich by-products can serve as inexpensive, non-caloric bulking agents for the partial replacement of flour, fat or sugar. They can also be used to enhance water and oil retention and to improve emulsion or the oxidative stability of food products [2,3]. Due to the increasing importance of these products in the food industry, several studies have addressed their characterisation, either of physicochemical properties [4,5] or composition in bioactive compounds [6,7]. Although aroma is a key sensory attribute to consider when using a product in the food industry, to the best of our knowledge, only one study has been devoted to the volatile composition of one by-product, namely apple [8].

Gas Chromatography-Mass Spectrometry (GC-MS) is the ideal analysis technique to characterise the composition of the volatile fraction of fibres derived from the juice industry since GC offers high separation power and MS useful spectra for compound identification and quantification. On the other hand, Solid-Phase Microextraction (SPME), introduced by Arthur and Pawliszyn [9] and extended to headspace (HS) sampling by Zhang and Pawliszyn [10], is a reliable routine technique to sample the volatile fraction of complex matrices because of its simplicity, sensitivity, possibility of automation and lack of solvent use. In HS-SPME, the analytes in the headspace of the investigated matrix are recovered onto a thin polymeric film coated on fused silica fibre and are then desorbed directly into the GC injector by thermal desorption. In addition to the selection of the most adequate fibre coating, several parameters such as sample amount, addition of salt saturated water (salting out effect), extraction time, temperature, and agitation rate need to be optimised to obtain a suitable method to characterise the volatile fraction of the fruit and vegetable fibre samples under investigation.

Enantioselective gas chromatography analysis (Es-GC) using cyclodextrines as chiral selectors has been applied in the quality control of several fruits and beverages to detect adulteration with synthetic flavours [11,12] and to monitor the possible effects of orange juice thermal processing on the enantiomeric ratio of several terpenic components [13]. Therefore, the study of the enantiomeric ratio of diagnostic chiral volatile compounds present in fibre samples can offer further useful information for their characterisation.

The aim of this work was to develop a HS-SPME-GC-MS method to characterise the volatile fraction of several fruit and vegetable matrices which play an important role in juice producing industries and are expected to be further applied as food ingredients resulting in a valorisation of what initially was considered as a residue. The fibres analysed included apple, pear, peach, carrot, lemon flesh, orange flesh, orange peel, tangerine flesh and tangerine peel. These fibres were obtained from several batches of processed industrial raw material from a currently operative juice production line.

Moreover, the composition of these fruit-derived by-products has been compared to the results of several existing studies reporting the volatile composition of raw fruits and juices to assess the differences between fruits and related fibres resulting from processing. In addition, an enantioselective analysis of some of the chiral terpenes present in the fruit fibre samples was performed and their enantiomeric ratios were compared to those reported in the literature, in order to determine possible variations caused by the processing to which the fruit was subjected in the juice industry.

2. Experimental

2.1. Samples

A local juice company (Indulleida S.A., Alguaire (Lleida), Spain) provided fibre samples from apple (6), pear (5), peach (5), carrot (1), lemon flesh (5), orange flesh (6), orange peel (1), tangerine flesh (1), and tangerine peel (1). All samples were industrially processed according to the scheme shown in Figure 1. This procedure involved washing with potable water followed by a wet milling. Next, samples were submitted to a drying step and milled again. Finally, fibres were sieved to achieve and homogenous texture and sacked.

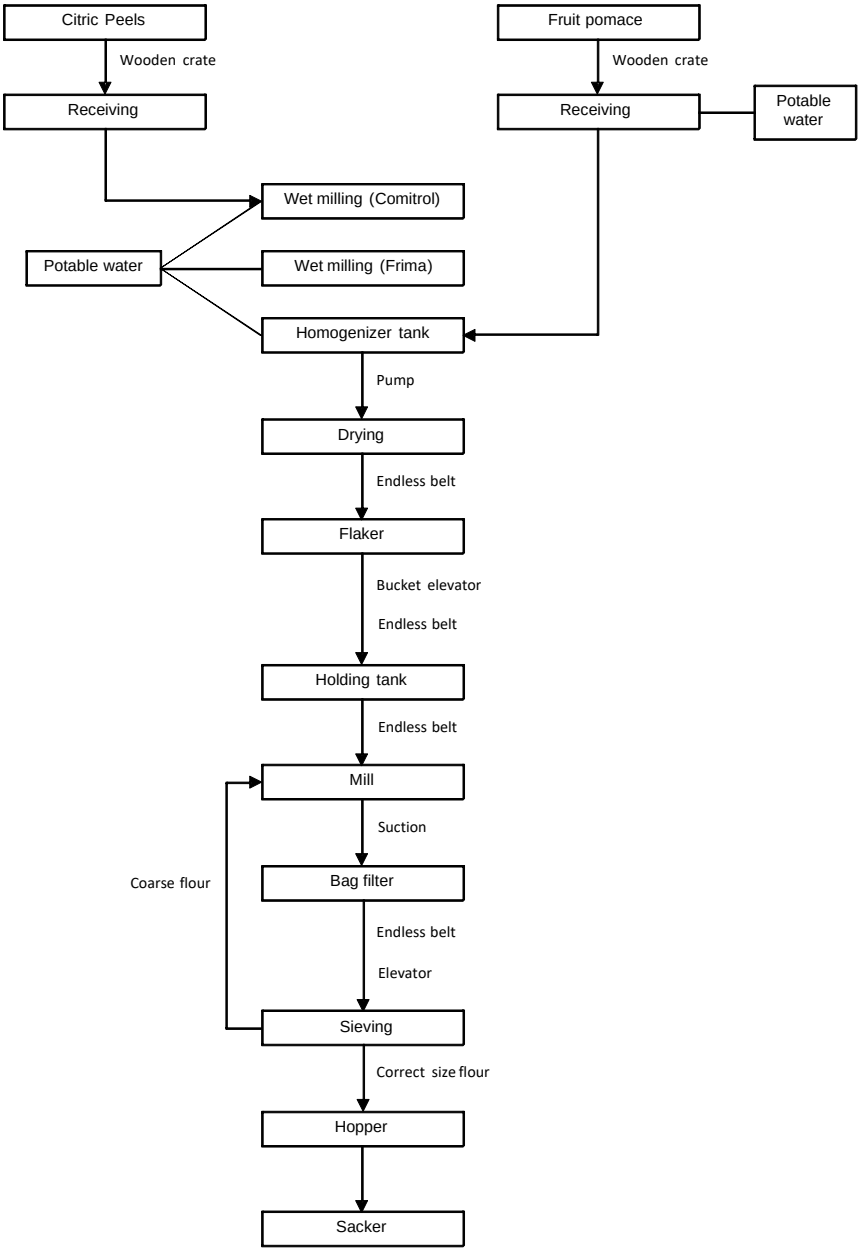

Figure 1: Schematics of the production process to obtain the analysed fruit fibres from residues of the juice industry.

2.2. Headspace Solid-Phase Microextraction (HS-SPME)

Between 100 mg and 1 g, depending on the sample, of fruit fibre were homogenised in 10 mL of H_2O saturated with NaCl and placed in a 20 mL headspace vial.

HS-SPME of the volatile fraction was carried out with a 2 cm triphase SPME fibre CAR/PDMS/DVB (Carboxen/ Polydimethylsiloxane/ Divinylbenzene; 50/30 µm) from Supelco (Bellefonte, PA, USA) at 50 ºC for 45 min using an agitation of 250 rpm.

2.3. Gas Chromatography-Mass Spectrometry (GC-MS) analysis

GC-MS analyses were performed with an MPS-2 multipurpose sampler (Gerstel, Mülheim an der Ruhr, Germany) assembled on an Agilent 6890 (Palo Alto, CA, USA) gas chromatograph coupled to an Agilent 5973N quadrupole Mass Selective Detector (MSD). The SPME fibre was desorbed into the injection port at 250 ºC in split mode (ratio 1:5) for 5 min. Compounds were separated with a MEGA5 column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) from Mega (Legnano, MI, Italy) using helium as carrier gas (1 $mL \cdot min^{-1}$). The oven was temperature programmed from 50 ºC (held for 1 min) to 160 ºC at 3 $ºC \cdot min^{-1}$, then to 250 ºC at 20 $ºC \cdot min^{-1}$ (held for 2 min). Mass spectra were recorded in electron impact (EI) mode at 70 eV within the mass range 35–350 m/z. The transfer line, the ionization source and the quadrupole were thermostated at 280, 230 and 150 ºC, respectively. Acquisition was done using MSD ChemStation software (Agilent Technologies, Palo Alto, CA, USA). All analyses were performed in duplicate.

Volatile compound identification was based on the comparison of experimental spectra with those of the Wiley 7 and Essential Oils mass spectral libraries (Wiley, New York, NY, USA), and was further confirmed by linear retention indices (LRI) calculated using an *n*-alkane mixture (*n*C9:*n*C30) [14], which were compared to those reported in Adams database [15] and Nist WebBook [16].

Peak areas calculated from total ion current (TIC) for each compound were normalised by in-fibre internal standardisation [17] as follows: 5 µL of 50 ppm solution of tridecane in dibutylphtalate was sampled for 15 min at 50 ºC and the relative abundance data (percentage on total volatile composition) were then calculated. This procedure was adopted to normalise the analytical deviation produced by variations in the performance of fibre and instrumentation [17].

2.4. Enantioselective-Gas Chromatography (Es-GC) analysis

Fruit fibres were manually sampled using the same conditions as described in section 2.2. The analyses were carried out on a Shimadzu GC-2010 system coupled to a

FID detector and controlled with Shimadzu GC Solution 2.30.00 software (Shimadzu, MI, Italy).

The SPME fibre was desorbed into the injection port at 220 ºC in split mode (ratio 1:5) for 5 min. Analyses were carried out on columns coated with 30% 2,3-di-*O*-ethyl-6-*O*-*tert*-butyldimethylsilyl-β-cyclodextrin (diEt-CD) diluted in PS-086 and 30% 2,6-di-methyl-3-*O*-pentyl-β-cyclodextrin (Pentyl-CD) diluted in PS-086, both from Mega (Legnano, MI, Italy), using hydrogen as carrier gas (1.25 mL·min^{-1}). The oven was temperature programmed from 50 ºC to 127 ºC at 1.87 ºC·min^{-1}; then to 220 ºC at 15 ºC·min^{-1} (held for 1 min). The chromatographic conditions were selected on the basis of the conditions used for the construction of the dedicated chiral library [18] and translated using the GC Method Translator Software (Agilent). LRI were calculated using a mixture of *n*-alkanes (*n*C9:*n*C30). The elution order of each enantiomer was assigned using a dedicated chiral library of racemic standards available in the laboratory [18].

3. Results and discussion

3.1. HS-SPME optimisation

CAR/PDMS/DVB SPME fibre was chosen for HS sampling due to its known capacity to efficiently recover compounds within an extended range of polarity [19]. Two matrices (lemon and peach) were chosen for optimisation. The parameters studied included amount of sample, use of NaCl saturated water to increase the ionic strength and reduce the water solubility of the compounds of interest, extraction time, temperature and stirring (0, 250, 500 and 750 rpm). The maximum amount of sample affording an appropriate homogenisation in 10 mL of NaCl saturated water was 1 g for samples containing low amounts of volatile compounds (non-citrus fibres). On the other hand, 0.5 g of flesh fibre and 0.1 g of peel fibre were used for citrus fruit samples due to their higher content of volatiles. Addition of NaCl-saturated water resulted in a 2-fold increase in total volatile area while extraction time and temperature only slightly influenced recovery in a reasonable time and temperature range (15–45 min and 40–60 ºC). A significant increase in the total area of volatile compounds was observed by applying agitation during SPME sampling (results not shown). On the basis of these results, the optimised sampling conditions were 50 ºC for 45 min and a stirring rate of 250 rpm.

3.2. Characterisation of the volatile fraction of fruit fibres

The HS-SPME-GC-MS method described above was used to characterise the volatile fraction of nine fruit fibres derived from processed industrial raw materials obtained from a juice production line. Volatiles were identified through their LRI and mass spectral data. As expected, the profile of the chromatograms revealed a high similarity between the

citrus samples, namely orange, orange peel, tangerine, tangerine peel and lemon. On the other hand, the volatile fraction of apple, pear, peach and carrot samples was relatively poor. Figure 2 shows the HS-SPME-GC-MS profile corresponding to lemon fibre. Peach and lemon fibres were used to evaluate the repeatability of the method. Five replicates were analysed for each fibre on various days, resulting in a satisfactory %RSD <11% for both fibres.

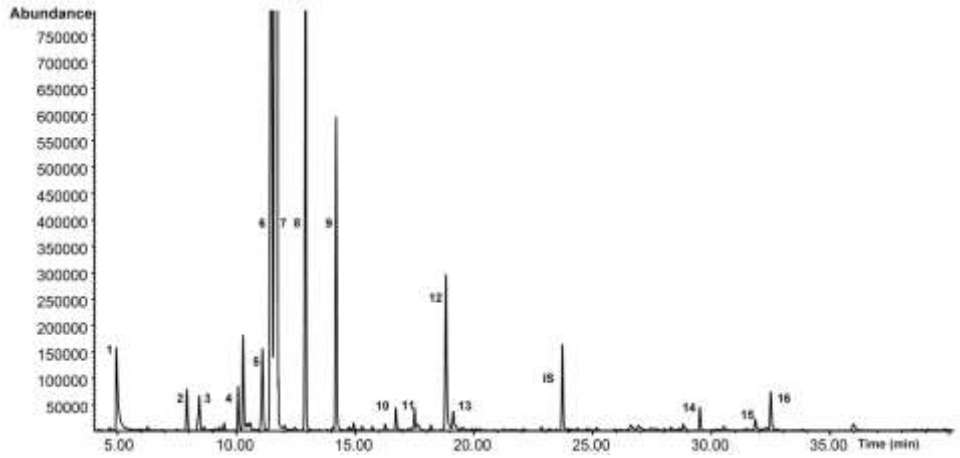

Figure 2: HS-SPME-GC-MS profile of a lemon flesh sample. Peak identification: (1) furfural, (2) α-pinene, (3) α-fenchene + camphene, (4) myrcene, (5) α-terpinene, (6) p-cymene, (7) limonene, (8) γ-terpinene, (9) α-terpinolene, (10) cis-β-terpineol, (11) terpinen-4-ol, (12) α-terpineol, (13) γ-terpineol, (14) *trans*-α-bergamontene, (15) valencene, and (16) β-bisabolene.

3.3. Volatile composition of citrus fibres

The volatile composition of citrus fibres (Table 1) consisted mainly of terpenoids, especially limonene, which accounted for about 52.7% of the total volatile fraction in lemon and over 90% in orange and tangerine fibres. Although limonene was the predominant volatile compound, all samples showed relatively high percentages of a large number of other terpenoids. For instance, lemon fibre contained, among others, 13.7% *p*-cymene, 7.4% γ-terpinene, 5.1% α-terpinolene, 4.7% α-terpineol and several other compounds at lower percentages.

Aldehydes accounted for 8.5% of the total volatile composition in lemon fibre, the most abundant of them being furfural, which probably derived from the decomposition of

sugars on the fibre. Other aldehydes found in lemon samples were heptanal, hexanal, (*E*)-2-heptenal, benzaldehyde and nonanal. Ketones, esters and alcohols were also found in the samples but at low concentrations (in all cases below 1%). Of note, the composition of the volatile fraction of citrus fibre is qualitatively comparable to those of raw fruits, essential oils [20] and juices [21-23].

Orange and tangerine flesh samples presented almost the same volatile composition, again showing a profile clearly dominated by terpenes (99.4 and 98.9% of total volatiles with a high predominance of limonene 92.3 and 94.0%, respectively). The same behaviour was observed for orange and tangerine peel fibres, which showed the same individual volatiles and similar percentages of the same. Moreover, the orange and tangerine peel samples presented a greater variety of compounds, including some terpenic acetates, (e.g. α-terpinyl, cytronellyl and neryl acetate) and sesquiterpenoids, such as β-cubebene, alloaromandrene, α-caryophyllene and α-(*E*)-ionone, which were not detected in the flesh samples.

On the basis of the total area, the residual amount of volatile fraction in tangerine and orange peel was higher than that in the corresponding flesh, the latter being much higher than the amount found in lemon. This finding is in agreement with previous studies that report a major content of volatile compounds, especially of limonene, in orange peel compared to orange flesh [24,25].

Table 1: Average relative percentage of volatile contents and their distribution ranges in different production batches (in parenthesis) of citrus fibres, as determined by HS-SPME-GC-MS analysis.

Compounds	LRI (exp)	LRI (ref)	Lemon fibre Mean (n=5)	Orange flesh fibre Mean (n=6)	Tangerine flesh fibre Mean (n=1)	Orange peel fibre Mean (n=1)	Tangerine peel fibre Mean (n=1)
Aldehydes							
Hexanal			0.2 (0.1-0.4)	0.1 (tr-0.2)	0.4	tr	tr
Furfural			7.7 (4.6-13.0)	tr	0.1		
Heptanal	902	905	tr	tr	0.1		
(*E*)-2-heptenal	959	957	0.2 (0.1-0.4)	0.1 (tr-0.1)	tr	tr	tr
Benzaldehyde	963	961	0.1 (0.1-0.1)	0.1 (tr-0.2)	0.3		
Nonanal	1106	1103	0.3 (0.2-0.4)	0.1 (tr-0.1)	0.2	tr	tr
Decanal	1207	1205		0.1 (tr-0.2)	0.1	0.2	tr
Subtotal			8.5	0.5	1.2	0.2	0.1
Ketones							
6-Methyl-5-hepten-2-one	989	985	0.1 (0.1-0.1)				
Subtotal			0.1	0.0	0.0	0.0	0.0
Esters							
Butyl isobutyrate	956	954	0.3 (0.1-0.5)	0.1 (tr-0.1)	0.1		
Hexyl butanoate	1194	1190				tr	tr
Octyl acetate	1216	1215		tr	0.1	0.1	0.1
Butyl benzoate	1378	1376	0.3 (0.2-0.4)				
Subtotal			0.6	0.1	0.2	0.1	
Alcohols							
1-Heptanol	973	970	0.1 (tr-0.2)				
Subtotal			0.1	0.0	0.0	0.1	0.1
Terpenoids							
α-Thujene	930	931				0.1	0.1
α-Pinene	937	939	0.7 (0.4-1.2)	0.4 (0.2-0.7)	0.4	0.7	0.9
α-Fenchene[1]	950	951	0.8 (0.6-1.3)				
β-Pinene[2]	978	980	0.1 (tr-0.2)	0.1 (tr-0.1)	tr	0.5	0.2
Myrcene	996	991	0.6 (0.4-1.4)	2.0 (1.4-3.4)	1.8	3.6	3.6
α-Phellandrene	1005	1005	0.2 (0.1-0.4)				
δ-3-Carene	1011	1011		0.2 (0.1-0.3)	0.1	0.2	tr
α-Terpinene	1017	1016	1.2 (0.5-3.6)	0.1 (0.1-0.2)	0.1	tr	tr

Table 1: (cont.).

Compounds	LRI (exp)	LRI (ref)	Lemon fibre Mean (n=5)	Orange flesh fibre Mean (n=6)	Tangerine flesh fibre Mean (n=1)	Orange peel fibre Mean (n=1)	Tangerine peel fibre Mean (n=1)
p-Cymene	1018	1018	13.7 (8.9-21.7)	0.6 (0.3-1.7)	tr	0.2	0.1
Limonene	1027	1026	52.7 (28.3-61.3)	92.3 (88.8-93.6)	94	92.2	90.4
β-Ocimene	1031	1031		0.1 (tr-0.2)	0.1	0.2	0.3
γ-Terpinene	1053	1050	7.4 (4.0-15.1)	0.5 (0.3-1.0)	0.4	0.4	2.6
α-Terpinolene	1062	1062	5.1 (3.7-9.3)	0.2 (0.2-0.3)	0.3	0.3	0.3
Linalool	1100	1098	0.1 (0.1-0.1)	0.1 (tr-0.1)	tr	tr	tr
1,3,8-p-Menthatriene	1113	1111		tr (tr-0.1)	tr	tr	tr
Endo-fenchol	1114	1112	0.2 (0.1-0.2)				
Unknown (MW=172)	1124		0.1 (0.1-0.2)	tr	tr	tr	tr
Terpinen-1-ol	1137	1134	0.1 (0.1-0.3)	tr	0.2	tr	tr
cis-β-Terpineol	1147	1144	0.5 (0.4-0.8)	tr	tr		
Borneol	1163	1165	0.5 (0.4-0.6)				
4-Terpineol	1178	1177	0.2 (0.1-0.2)	0.1 (0.1-0.3)	tr	0.1	0.1
α-Terpineol	1191	1189	4.7 (3.2-6.6)	0.3 (0.1-0.5)	0.2	tr	tr
γ-Terpineol	1196	1192	0.4 (0.2-0.9)				
Safranal	1199	1201		tr	0.1	tr	tr
Carvone	1250	1245		tr	0.1	tr	tr
α-Terpinyl acetate	1356	1350				0.1	tr
Cytronellyl acetate	1361	1360				tr	0.1
Neryl acetate	1371	1368				tr	0.1
α-Copaene	1379	1376		0.2 (0.1-0.3)	0.3	0.1	0.1
β-Elemene	1393	1391				0.1	0.1
(*E*)-b-caryophyllene	1420	1418		0.1 (tr-0.1)	tr		
α-(*E*)-ionone	1428	1426				0.1	tr
β- Cubebene	1430	1434				tr	tr
trans-α-Bergamotene	1437	1438	0.4 (0.2-0.8)				
Alloaromandrene	1452	1455				tr	tr
α-Caryophyllene	1453	1455				tr	tr
β-Farnesene	1459	1458		0.2 (0.1-0.4)		tr	tr
β-Ionone	1488	1485					
Valencene	1493	1491	0.5 (0.2-0.7)	1.6 (0.1-4.1)	0.1	0.8	0.7
β-Bisabolene	1510	1509		0.1 (tr-0.4)	0.5	tr	0.1
α-Farnesene	1511	1508			0.1		

Table 1: (cont.).

Compounds	LRI (exp)	LRI (ref)	Lemon fibre Mean (n=5)	Orange flesh fibre Mean (n=6)	Tangerine flesh fibre Mean (n=1)	Orange peel fibre Mean (n=1)	Tangerine peel fibre Mean (n=1)
7-epi-α-Selinene	1516	1517		0.1 (tr-0.2)	tr	tr	tr
δ-Cadinene	1525	1524		0.1 (tr-0.1)	0.1	0.1	0.1
γ-Bisabolene	1534	1533	0.6 (0.3-1.4)				
Total (%)			91	99.4	98.9	99.8	99.9
Total normalised area			30.6±9.7	68.1±42.7	65.1	408.7	353.3

[1] In lemon fibre, area includes camphene as a result of coelution.

[2] In orange and tangerine peel fibres, area includes sabinene as a result of coelution.

Traces (tr): indicates area percentage <0.05%.

3.4. Volatile composition of non-citrus fruit and carrot

Unlike citrus fibre, apple, pear, peach and carrot fibres showed a volatile composition with lower percent of terpenoids (Table 2). In this case, the analyses revealed that the most abundant group of compounds in apple fibre was that of aldehydes (57.5%), the main ones being hexanal (19.7%), benzaldehyde (15.6%) and (*E*)-2-heptenal (14.9%). Esters accounted for 16.3% of the volatile fraction, with butyl isobutyrate (12.1%) as the major component. Also, ketones were present in a considerable amount (11.6%), while terpenoids accounted for 11.4%.

The volatile fraction composition of apple fibre was severely affected during fibre production if compared to that of raw fruit described in several publications [26,27]. This observation could be attributed to the thermal treatment used during the juicing process. Former studies report ethyl esters, higher alcohols, and α-farnesene as the main components rather than aldehydes.

Pear fibre contained esters as the main constituents (54.0%), hexyl acetate being the most abundant (49.1%). Volatile aldehydes accounted for a substantial fraction of these samples (32.8%), the most abundant being furfural (15.2%), followed by hexanal, (*E*)-2-heptenal, benzaldehyde, octanal and heptanal. Other groups of compounds, such as alcohols, ketones, ethers and terpenoids, were present in minor percentage. In this case, the volatile fraction of pear fibre is qualitatively comparable to that of raw fruits reported in previous studies [28], where esters were found to be the main fraction. Riu-Aumatell et al. [29] reported hexyl acetate as one of the compounds consistently found in 11 commercial samples of pear juice.

Peach fibre also showed a high proportion of aldehydes (69.7%), where furfural (43.2%) and hexanal (17.4%) prevailed, together with heptanal, benzaldehyde, (*E*)-2-

heptenal and nonanal in percentages ranging on average between 1.4 and 2.6%. For these samples, terpenoids accounted for 22.4% of the volatile fraction. The main terpenoids found in peach fibre were mainly α-terpineol, limonene, and α-phellandrene. Ketones and ethers were present in lower percentages, 6.1 and 1.6%, respectively. The volatile fraction of the peach fibres contained several terpenoids at a percentage comparable to that of raw fruits [29], while lactones, key markers of peach aroma [30,31], were not detected.

The volatile fraction of carrot fibre contained terpenoids as the main group of compounds (35.3%). Other studies have reported that these compounds account for 97% of the total volatile fraction of fresh carrot samples [32]; the lower percent found in the analysed sample could be explained by the loss of volatiles during the washing and drying treatment applied during industrial fibre processing. The most abundant components of carrot fibre were α- and β-ionone, at 8.1 and 9.8%, respectively. The correlation between carotenoid degradation caused by processing and the production of degradative terpenes such as ionones has been described by Kanasawud and Crouzet [33]. Aldehydes accounted for 32.8% of total volatile composition of this fibre, with hexanal at 20.3% and ketones at 16.1%. These included 1-octen-3-one, 6-methyl-5-hepten-2-one, 2-methyl-3-octanone, 2,2,6-trimethylciclohexanone, and 2,3,4-trimethylcyclohexen-1-one, all present at between 1.9 and 6.5%. Esters, ethers, and alcohols were present at 5.1, 1.8 and 1.3%, respectively. On the basis of the total area, the volatile fractions of non-citrus fibre was about 10-fold lower than citrus flesh fibre and almost 100-fold lower than citrus peel fibre, i.e. the matrices containing the highest amount of volatile compounds.

Table 2: Average relative percentage of volatile compounds present and their distribution ranges in different production batches (in parenthesis) of apple, pear, peach and carrot fibres, as determined by HS-SPME-GC-MS analysis.

Compounds	LRI (exp)	LRI (ref)	Apple fibre Mean (n=6)	Pear fibre Mean (n=5)	Peach fibre Mean (n=5)	Carrot fibre Mean (n=1)
Hydrocarbons						
2,4-Dimethyl-1-heptene			2.1 (0.9-4.4)	0.1 (tr-0.3)		
4-Methyl octane			0.5 (tr-1.3)			3.0
Subtotal			2.6	0.1	0.0	3.0
Aldehydes						
Hexanal			19.7 (9.2-27.7)	7.4 (5.2-9.0)	17.4 (10.6-21.3)	20.3
Furfural			2.6 (1.8-3.4)	15.2 (9.0-21.1)	43.2 (38.8-43.3)	1.6
Heptanal	902	905	1.3 (1.0-1.7)	0.7 (0.5-0.9)	1.4 (0.5-1.7)	2.6
(*E*)-2-heptenal	959	957	14.9 (12.1-20.2)	3.9 (1.5-6.4)	2.6 (1.8-4.1)	3.6
Benzaldehyde	963	961	15.6 (11.6-22.4)	3.6 (0.9-6.3)	2.5 (tr-3.6)	1.3
Pentyl furane	993	996	tr			
Octanal	1004	1001	1.7 (1.0-3.3)	2.0 (1.34-2.41)		
Nonanal	1106	1103	1.7 (1.2-2.2)		2.6 (tr-4.1)	3.0
Decanal	1207	1205				0.4
Subtotal			57.5	32.8	69.7	32.8
Ketones						
1-Octen-3-one	980	980	6.3 (3.9-9.2)	3.1 (0.9-4.4)	1.7 (1.0-3.0)	4.7
2-Methyl-3-octanone	986	985			0.7 (tr-1.8)	
6-Methyl-5-hepten-2-one	989	985	5.3 (2.7-7.7)	2.7 (1.9-3.7)	3.7 (2.50-4.8)	3.0
2,2,6-Trimethylciclohexanone	1037	1036				6.5
3,4,4-Trimethylcyclohexen-1-one	1082					1.9
Subtotal			11.6	5.8	6.1	16.1
Ethers						
Butyl ether			0.7 (0.4-1.0)	1.0 (0.4-1.4)	1.6 (tr-2.2)	1.8
Esters						
Butyl acetate				3.8 (2.9-6.0)		
Pentyl acetate	917	916		1.1 (0.9-1.2)		
Butyl isobutyrate	956	954	12.1 (9.5-16.6)			0.9
Hexyl acetate	1017	1016	1.5 (0.5-2.7)	49.1 (40.4-61.2)		
Hexyl 2-methylbutanoate	1244	1239	0.6 (0.2-1.1)			
Butyl benzoate	1378	1376	1.3 (1.1-2.0))			4.2
Hexyl hexanoate	1391	1386	0.8 (0.3-2.7)			
Subtotal			16.3	54	tr	5.1

Table 2: (cont.).

Compounds	LRI (exp)	LRI (ref)	Apple fibre Mean (n=6)	Pear fibre Mean (n=5)	Peach fibre Mean (n=5)	Carrot fibre Mean (n=1)
Alcohols						
1-Hexanol				1.2 (0.8-1.7)		
(E)-2-cyclohexen-1-ol	1097	1097				0.9
2,6-Dymethyl cyclohexanol	1110	1114				0.4
Subtotal			tr	1.2	tr	1.3
Terpenoids						
α-Pinene	937	939	0.2 (tr-0.5)		0.2 (tr-0.6)	1.2
β-Pinene	978	980		1.2 (0.6-2.3)		
Myrcene	996	991				0.6
α-Phellandrene	1005	1005			3.2 (1.8-6.1)	
p -Cymene	1027	1026			1.1 (0.3-2.0)	2.7
Limonene	1031	1031	3.1 (0.9-3.2)		6.1 (1.9-17.8)	
γ-Terpinene	1062	1062	5.6 (4.0-8.9)		2.2 (0.4-3.4)	
α-Terpinolene	1086	1088				1.3
Linalool	1100	1098			0.9 (0.5-2.0)	
cis-β-Terpineol	1147	1144	0.2 (0.1-0.3)			
4-Terpineol	1178	1177			0.4 (tr-1.3)	
α-Terpineol	1191	1189	1.5 (0.8-2.0)	1.5 (1.0-1.9)	6.6 (4.1-9.5)	1.0
β-Cyclocitral	1223	1222				3.5
Neryl acetate	1371	1368			1.1 (0.6-1.5)	
(*E*)-β-caryophyllene	1420	1418				1.9
α-Ionone	1428	1426				8.1
Geranyl acetone	1454	1455				2.5
β-Ionone	1488	1485				9.8
Valencene	1493	1491	0.4 (0.1-0.5)		0.2 (tr-0.5)	
α-Farnesene	1511	1508	0.4 (0.2-0.8)	0.2 (0.2-0.3)		
7-epi-α-Selinene	1516	1517			0.4 (tr-2.1)	
γ-Bisabolene	1534	1533				2.7
Total (%)			11.4	2.9	22.4	35.3
Total normalised area			7.7±2.4	5.0±1.5	4.1±2.3	5.6

Traces (tr): indicates area percentage <0.05%.

3.5. Es-GC analysis of chiral markers in fruit fibre samples

Here we sought to study some of the chiral markers present in the fruit fibre samples in order to assess whether the processing (which includes thermal treatment) affects the enantiomeric ratio (ER), i.e. with an increase of racemisation of some chiral compounds. HS sampling by SPME was therefore applied in the same optimised conditions as previously described in section 3.1, in combination with Es-GC with cyclodextrin derivatives as chiral selectors. The ER of the selected chiral markers was compared to those previously reported in the literature for samples of the same fruit origin, namely fresh fruits, juices or essential oils, when available.

Only five chiral markers could be selected (α-pinene, β-pinene, limonene, α-terpineol and α-ionone) for non-citrus fibres due to the low abundance of volatile compounds, as reported in section 3.2.1. On the other hand, for citrus samples, chiral marker selection was limited by the presence of coelutions. DiEt-β-CD and Pentyl-β-CD columns were used to achieve a reliable separation of a higher number of compounds. Moreover, pure standard mixtures of racemic terpenes were injected under the same Es-GC conditions to facilitate enantiomer identification.

3.6. Es-GC analysis of selected chiral markers in non-citrus fruit and carrot fibres

Very few chiral compounds were analysed in non-citrus fibres. However, the ER variability of chiral compounds among samples of each fruit was low (Table 3). A high ER was measured for the *R*-limonene enantiomer (> 99%). α-Pinene and α-ionone in carrot fibre were present with a higher ER in favour of the S enantiomer, while β-pinene in pear was present in racemic form. α-Terpineol was found in all samples, with a higher abundance of the *S*-enantiomer, ranging from 58.0 to 74.8%, in all non-citrus samples.

Table 3: Chiral markers, calculated LRI and corresponding enantiomeric ratio, for non-citrus fruit fibre.

Chiral marker	Configuration	LRI	Apple (n=6)	Pear (n=5)	Peach (n=5)	Carrot (n=1)
α-Pinene	S	923				69.7
	R	925				30.3
β-Pinene	R	946		47.5-51.9		
	S	956		52.5-48.1		
Limonene	S	1056	tr		tr	
	R	1072	>99.9		>99.9	
α-Terpineol	R	1296	39.5-46.6	40.6-41.3	35.3-42.0	25.2
	S	1309	60.5-63.4	59.4-58.7	64.3-58.0	74.8
α-Ionone	R	1414				12.6
	S	1424				87.4

3.7. Es-GC analysis of selected chiral markers in citrus fruit fibre

ERs were calculated for eight chiral markers in lemon fibre (Table 4). The results are, in general, in good agreement with those reported for the enantiomeric composition of essential oils. An ER was observed for all the chiral markers except for linalool, which was almost in racemic form. This result is in agreement with the literature reporting that the enantiomeric composition of linalool in lemon essential oils is highly variable depending on the cultivar and harvest period [34]. The monoterpenes α-pinene, β-pinene, borneol, and α-terpineol presented a higher ratio of the *S*-enantiomer while camphene and limonene gave higher ratios of the *R*-enantiomer. The ERs calculated for this compounds show in all cases the same predominance of one of the enantiomers as reported in the literature [35]. However, the ER of terpinen-4-ol tended to vary as a consequence of the high temperatures applied during processing: the pre-treatment of the lemon fibres at high temperatures might explain a lower ER of the *R*-enantiomer in these fibres when compared with essential oils and juices [36].

Table 4: Chiral markers, calculated LRI and corresponding enantiomeric ratio, for lemon fibre.

Chiral marker	Configuration	LRI	Lemon (n=5)	Literature data [35]
α-Pinene[1]	R	923	15.4-22.0	25.5-37.8
	S	925	84.6-78.0	74.5-62.2
Camphene[1]	S	920	72.6-80.7	86.2-92.4
	R	933	27.4-19.3	13.8-7.6
β-Pinene[1]	R	946	15.8-21.3	4.2-7.0
	S	956	84.2-78.7	95.8-93.0
Limonene[1]	S	1056	0.9-4.2	1.0-2.6
	R	1072	99.1-95.8	99.0-97.4
Linalool[2]	R	1212	38.0-49.7	49.5-74.5
	S	1222	62.0-53.3	50.5-25.5
Borneol[2]	S	1307	84.5-91.7	
	R	1317	15.5-8.3	
Terpinen-4-ol[2]	S	1319	30.0-43.0	12.0-32.5
	R	1327	70.0-57.0	88.0-67.5
α-Terpineol[1]	R	1296	11.0-18.5	35.8-18.0
	S	1309	89.0-81.5	64.2-82.0

[1] LRI and enantiomeric ratios calculated using a diEt-CD column.

[2] LRI and enantiomeric ratios calculated using a Pentyl-CD column.

ERs were calculated for nine chiral markers in orange and tangerine fibres (Table 5). These results are generally in good agreement with the literature on citrus essential oils [35], often showing a higher ER for one of the enantiomers, as was the case for α-pinene, camphene, limonene, linalool and carvone. β-Pinene, as previously described, presented a high ER of *S*-enantiomer in orange and tangerine flesh fibres, while it was racemic in both peel fibres [35]. In agreement with the reported data, the drying process applied to the fibres is expected to have modified the ER of terpinen-4-ol and α-terpineol. A similar effect had already been reported for these monoterpene alcohols when citrus essential oils are obtained through distillation instead of cold pressing [35]. Finally, the ERs of α-terpineol in orange and tangerine peel were not coincident and showed distinct behaviour. This difference was also observed in the ER of α-terpinyl acetate. This observation could be explained by the fact that α-terpinyl acetate forms from α-terpineol via acetylation. On the other hand, for tangerine peel, racemisation was observed for α-terpineol, while α-terpinyl acetate probably kept its original configuration.

Table 5: Chiral markers calculated LRI, and corresponding enantiomeric ratios, for orange and tangerine fibres.

Chiral marker	Config.[3]	LRI	Orange flesh (n=5)	Tangerine flesh (n=1)	Orange peel (n=1)	Tangerine peel (n=1)	Literature data [35]
α-Pinene [2]	S	929	7.6-17.8	13.4	11.7	14.5	9.9-0.6
	R	936	92.4-82.2	86.6	88.3	85.5	90.1-99.4
Camphene[2]	S	949			29.2	25.4	
	R	964			70.8	74.6	
β-Pinene[2]	R	975	6.5-12.6	6.1	46.2	49.3	10.6-70.2
	S	978	93.5-87.4	93.9	53.8	50.7	89.4-29.8
Limonene[1]	S	1056	0.6-0.8	0.6	0.5	0.6	0.0-1.1
	R	1072	99.4-99.2	99.4	99.5	99.4	100-98.9
Linalool[1]	R	1175	9.7-20.4	16.4	8.1	11.3	2.2-17.9
	S	1190	90.3-79.6	83.6	91.9	88.7	97.8-82.1
Terpinen-4-ol[2]	S	1319	42.9-51.1	28.9	55.7	57.8	65.3-71.5
	R	1327	57.1-49.9	71.1	44.3	42.1	34.7-28.5
α-Terpineol[1]	R	1296	30.6-42.1	41.5	22.7	48.3	5.1-15.7
	S	1309	69.4-57.9	58.5	77.3	51.7	94.9-84.3
Carvone[2]	R	1346	32.2-41.3	34.9	30.3	26.5	40.7
	S	1352	67.8-58.7	65.1	69.7	73.5	59.3
α-Terpinyl	X	1378			21	73.5	
acetate[2]	Y	1381			79.2	26.5	

[1] LRI and enantiomeric ratios calculated using diEt-CD column.

[2] LRI and enantiomeric ratios calculated using Pentyl-CD column.

[3] X and Y were used to indicate that the absolute configuration of the enantiomers could not be determined.

4. Conclusions

Here we applied HS-SPME-GC-MS to study the volatile composition of nine types of fruit and vegetable fibres, namely apple, peach, pear, orange peel and flesh, tangerine peel and flesh, lemon flesh and carrot, derived from the juice industry. Although being submitted to a processing which involves among others washing and drying this study shows that the volatiles remaining in the fibres cannot be neglected. In this regard, citrus fibres contained a high amount of volatile compounds, mainly monoterpenoids (limonene). The processing to which fruit residues to obtain fruit fibres showed to produce

fibres with low volatile content for non-citrus products. Otherwise, citrus fibres analysed still presented a high volatile composition when compared with non-citrus. In addition, the Es-GC analyses of the chiral volatiles present in the samples revealed that during processing, monoterpene alcohols, (terpinen-4-ol and α-terpineol) tend to show a variation in their ER, probably because of the heat applied during drying.

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3.3.2. Development of a SBSE-TDU method coupled to GC-MS and chemometrics for the differentiation of variety and processing conditions in peach juices

DEVELOPMENT OF A SBSE-TDU METHOD COUPLED TO GC-MS AND CHEMOMETRICS FOR THE DIFFERENTIATION OF VARIETY AND PROCESSING CONDITIONS IN PEACH JUICES

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Abstract

Here we describe a method based on Stir Bar Sorptive Extraction (SBSE) involving a polydimethylsiloxane-coated stir bar with thermal desorption (TD), followed by gas chromatography coupled to mass spectrometry (GC-MS) analysis. The method was applied to distinguish peach juices of distinct varieties, namely yellow- and red-fleshed, and between two processing conditions—commercial and freshly blended. The resulting analytical data included 41 compounds belonging to several chemical classes, such as aldehydes, alcohols, lactones, terpenoids, fatty aldehydes, fatty acids and hydrocarbons. Furthermore, chemometric data treatment using unsupervised analysis (PCA) proved useful to classify peach juices on the basis of variety. Stepwise Linear Discriminant Analysis (SLDA) showed that a reduced number of variables (14 compounds), including lactones (6-pentyl-α-pyrone, γ-decalactone, γ-dodecalactone, and δ-dodecalactone), fatty acids (hexadecanoic acid), fatty aldehydes (tetracosanal and octacosanal), hydrocarbons (C23, C26, C27, C29, and C33), and alcohols (phytol and α-tocopherol), were necessary to classify the juice samples according to variety and processing conditions.

Keywords: *SBSE, GC-MS, PCA, SLDA, peach juices.*

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1. Introduction

Fruit juice production and distribution is an important activity in the European agro-industrial economy, as reflected by the consumption of $9.7x10^9$ L of fruit juice in Europe alone in 2014 [1]. Among these juices, peach juice ranks high in terms of consumption, falling only behind orange, pineapple and apple [2]. Although the aroma of juice is given by a complex set of volatile substances, a group of about 25 appear to define the typical peach aroma. In particular, γ- and δ-decalactone play a key role, in association with C6 compounds, alcohols, esters, terpenoids, and phenolic volatiles [3]. Aroma is one of the main attributes to be considered with regard to consumer acceptance. In addition, the volatile composition of a juice can be affected by several variables, including variety [4], climatological conditions [5], and technological variables such as storage and processing procedures [4, 6, 7].

Gas chromatography-mass spectrometry (GC-MS) is a suitable technique to determine the volatile and semi-volatile composition of a juice, since GC offers high separation power and MS useful spectra for compound identification and quantification. However, prior to GC analysis, it is necessary to extract these compounds from the aqueous matrix. Several techniques can be used for this purpose. In this regard, in recent years, special attention has been paid to microextraction techniques, which require minimum amounts of solvent or no solvent. Single Drop Microextraction (SDME) [8], Solid Phase Microextraction (SPME) [9], and Stir Bar Sorptive Extraction (SBSE) [10] are examples of such techniques. With regard to SDME, when agitation is applied, it may lack drop stability when samples are extracted directly from the aqueous phase instead of the headspace (HS) [11]. Therefore, SDME is used almost exclusively for very clean samples, such as tap water and groundwater [12]. In the case of SPME, although it is sometimes used in immersion mode [13, 14], direct coating exposure in a complex matrix may lead to extraction-phase fouling and subsequent loss of extraction sensitivity, reproducibility, accuracy, and extract integrity [15]. In contrast, SBSE was specially conceived for the analysis of aqueous samples in immersion mode. Indeed, the first application of this technique, which was developed by Baltussen et al., was the analysis of micro-pollutants in aqueous samples [10]. However, applications to HS sampling have also been reported [16].

The characterization and differentiation of variety or origin of fruits and derived products by analysis of their volatile composition have been widely reported. In this regard, most research has been devoted to the analysis of the HS with either SPME [5, 17, 18] or, to a lesser extent, SBSE [19]. However, it is difficult to find research dealing with the direct analysis of fruit juice—a matrix that contains both volatiles and semi-volatiles. Indeed, the most remarkable applications of this technique to aqueous matrices have been for coffee [16], whisky [20], being also applied in solid matrices like raw fruit [21, 22]. However, there are no reports of the application of SBSE-TD to the analysis of peach juices of distinct varieties and subjected to different processing conditions.

Multivariate techniques are often, and increasingly, applied in chromatographic data analysis to deal with the considerable number of samples and variables involved.

Among the wide variety of multivariate methods available, Principal Component Analysis (PCA) is one of the most common unsupervised techniques used, while Stepwise Linear Discriminant analysis (SLDA) is frequently applied as a supervised technique for sample classification purposes.

Here we sought to develop a SBSE-TD-GC-MS method to analize the volatile and semi-volatile composition of yellow- and red-fleshed peach varieties and of industrially processed and freshly squeezed peach juice. PCA an SLDA were applied to distinguish between these juices. To the best of our knowledge, this is the first study to apply a SBSE-TD-GC-MS method to the analysis of volatile and semi-volatile compounds in peach juice for the differentiation of fruit variety (agronomic factor) and distinction between industrially processed and non-processed juice (technological factor).

2. Materials and methods

2.1. Peach juice samples

Six commercial peach (*Prunus persica*) juices of Spring Lady and Miraflores cultivars and their corresponding fresh fruit were provided by a local 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 during blending. Peaches and the corresponding juices were stored at 4 ºC. Prior to extraction, juice samples were kept at room temperature for 1 h.

2.2. Stir Bar Sorptive Extraction (SBSE)

Commercial stir bars coated with a 0.5 mm-layer of poly(dimethylsiloxane) (PDMS) (10 mm x 0.5 mm film thickness 24 µL phase volume) and ethylene glycol-silicone copolymer (EG-Silicone) (10 mm length and 32 µL phase volume) were obtained from Gerstel (Mullheim an der Ruhr, Germany). Stir bars were conditioned prior to use following the manufacturer's instructions.

We introduced 10 mL of peach juice diluted with water (1:1) and 500 mg of NaCl into a 20-mL vial containing a PDMS-coated stir bar. Subsequently, 10 µL of a 1 µg/mL solution of 3-octanol in methanol (Sigma, Buchs, Switzerland) was added as internal standard. The vial was sealed and stirred at 800 rpm for 2 h at room temperature. The stir bar was removed from the solution, rinsed with distilled water, dried with lint-free paper, and immediately transferred to a thermal desorption tube. All analyses were performed in triplicate.

2.3. GC-MS

Thermal desorption was conducted with a TDU unit equipped with a multipurpose autosampler (MPS-2) from Gerstel installed on an Agilent 6890 GC (Agilent Technologies, Palo Alto, CA, USA) coupled to a Pegasus 4D ToF MS (Leco Corp., St. Joseph, MI, USA) system. The following parameters were used for the TDU: desorption program, from 30 to 265 °C (5 min) at 350 °C/min; carrier gas (helium) flow rate, 80 mL/min. A Gerstel CIS-4 PTV injector was used for cryogenic focusing of the analytes thermally desorbed from the stir bar. The PTV was cooled at 15 °C using a peltier UPC^{plus} unit from Gerstel, and the injection temperature was raised to 275 °C (5 min) at 10 °C/s. Capillary GC-MS analyses were performed using a DB-5MS (Agilent) column (30 m x 0.25 mm x 0.25 µm). Chromatographic conditions were as follows: solvent vent injection (20 s at 50 mL/min); temperature program: from 50 °C (0.5 min) to 240 °C at 10 °C/min and to 330 ºC (1 min) at 20 ºC/min; carrier gas, He; flow rate, 2 mL/min. A mass spectrometer with a time of flight (ToF) detector was used for detection. Mass spectra were recorded in electron ionization (EI) mode at 70 eV. The transfer line and the ion source were set at 250 ºC and 200 ºC, respectively. Mass spectra were scanned in the range *m*/*z* 40-400 amu. Compound identification was carried out by comparison of linear retention indices (LRI) calculated using an n-alkane mixture (*n*C9: *n*C30) [23]. Identifications were then compared to those reported in Adams Library [24] and Nist WebBook [25]. Mass spectrometry data and authentic standards were also used when available. Peak areas of the ions extracted for each compound (Table 1) were used for area quantification and were normalized using 3-octanol as internal standard.

Table 1: Compounds detected in the peach juice samples using the SBSE-TD-GC-MS methodology.

Peak num.	Compound	Code	LRI	Quantification Ion (m/z)
	Internal standard			
1	3-octanol	IS	994	112
	Aldehydes			
2	decanal	Ad2	1187	82
4	5-HMF	Ad4	1235	97
	Lactones			
3	γ-hexalactone	L3	1050	85
6	γ-octalactone	L6	1268	85
9	6-pentyl-α-pyrone	L9	1460	166
10	γ-decalactone	L10	1473	85
11	δ-decalactone	L11	1493	99
12	hydroxy lactone [a]	L12	1550	85
13	(*Z*)-6-γ-dodecenolactone	L13	1623	85
14	γ-dodecalactone	L14	1678	85
15	δ-dodecalactone	L15	1735	99
	Alcohols			
5	nerol	Al5	1239	93
17	3-octen-2-ol	Al17	1876	110
21	phytol	Al21	2113	71
38	α-tocopherol	Al38	3128	165
	Terpenoids			
8	geranyl acetone	T8	1430	116
	Fatty aldehydes			
26	docosanal	Fad26	2476	82
30	tetracosanal	Fad30	2639	82
31	pentacosanal	Fad31	2741	82
34	hexacosanal	Fad34	2844	82
39	octacosanal	Fad39	3046	82
	Fatty acids			
7	nonanoic acid	Fac7	1278	115
16	tridecanoic acid	Fac16	1685	219
19	hexadecanoic acid	Fac19	1921	129
22	unsaturated C18 acids [b]	Fac22	2153-2163	79

Table 1: (cont.).

Peak num.	Compound	Code	LRI	Quantification Ion (m/z)
	Hydrocarbons			
18	C19	H18	1900	71
20	C21	H20	2100	71
23	C22	H23	2200	85
24	C23	H24	2300	71
25	C24	H25	2400	71
27	C25	H27	2500	85
28	C26	H28	2600	85
29	C27	H29	2700	85
32	C28	H32	2800	99
33	squalene	H33	2811	95
35	C29	H35	2900	85
36	C30	H36	3000	85
37	C31	H37	3100	85
40	C32	H40	3200	85
41	C33	H41	3300	85

[a] Non-identified hydroxy lactone.

[b] Unresolved peaks corresponding to (9*Z*)-octadecenoic acid + (9Z, 12Z)-octadecadienoic acid.

2.4. Statistical analysis

Principal Component Analysis (PCA) and Stepwise Linear Discriminant Analysis (SLDA) of analytical data were performed with JMP Pro 12.0.1 software (SAS Institute, Cary, NC, USA). All compounds were included in the PCA, which is an unsupervised technique that reduces the dimensionality of the data set but retains the maximum amount of variability [26].

SLDA is a supervised method applied for classification purposes. SLDA classification was achieved by applying a forward stepwise linear variable selection and using the F-ratio to determine the most significant variables when a new variable was selected. The prediction capacity of the discriminant model was studied by cross validation of samples not included in the construction of the model.

3. Results and discussion

Compound extraction and thermal desorption are the two main steps in SBSE-TD. The first step includes the immersion of the polymer-coated stir bar into the HS vial that contains the liquid sample until equilibrium is reached. The second involves thermal desorption of the analytes accumulated on the surface of the bar into the chromatograph, using a TD unit in the case of GC.

3.1. SBSE optimization

Initially, the two commercially available phases, namely PDMS and EG-silicone copolymer, were tested. Given that these phases have distinct polarities, we studied their behavior with respect to the compounds extracted from a peach juice sample. Preliminary results showed that the PDMS coating extracted a higher number of compounds. In addition, EG-silicone presented carry-over effects for some compounds. These compounds were difficult to desorb due to their higher polarity and the lower desorption temperature recommended by the Twister manufacturer for this phase. On the basis of our results, EG-silicone was discarded for sample analysis and further optimization work.

Prior to the extraction, juice samples were diluted to reduce viscosity and facilitate homogenization. For this purpose, they were diluted 1:1 to achieve satisfactory homogenization without excessive dilution of the compounds, which would lead to a decrease in the analyte concentration determined and the number of compounds detected. Extraction time, modification of the ionic strength, and stirring speed were the variables studied for the extraction step. SBSE extraction conditions were optimized using a randomly selected sample of juice. The total area obtained under each of the extraction conditions was considered when selecting the optimum parameters to carry out further sample analyses.

Extraction time was studied from 1 to 6 h to achieve the maximum overall response in the minimum time. Extraction was maximized after 2 h, and an increase in the extraction time did not improve the results (Figure 1a). Thus, 2 h was the time required for the compounds in the liquid phase and the surface of the stir bar to reach equilibrium. Modification of the ionic strength (Figure 1b) by adding NaCl (0-10%, w/w) was also tested. It is well known that salting-out effect reduces the solubility of compounds in the aqueous phase, thus improving extraction by the PDMS phase. On the other hand, an excessive concentration of salts is expected to reduce the extraction efficiency, probably due to an increase in the viscosity of the solution. We observed that a concentration range from 2 to 5% NaCl (w/w) maximized the total area for the compounds in the samples. Finally, 5% NaCl was chosen to assure the maximum recovery in all cases. Considering the stirring rate (Figure 1c), the most remarkable effect was the use or not of agitation during the extraction. Given that no statistical differences were observed when agitation

rates were increased, an intermediate value was chosen to prevent excessive mechanical damage to the coating of the stir bar.

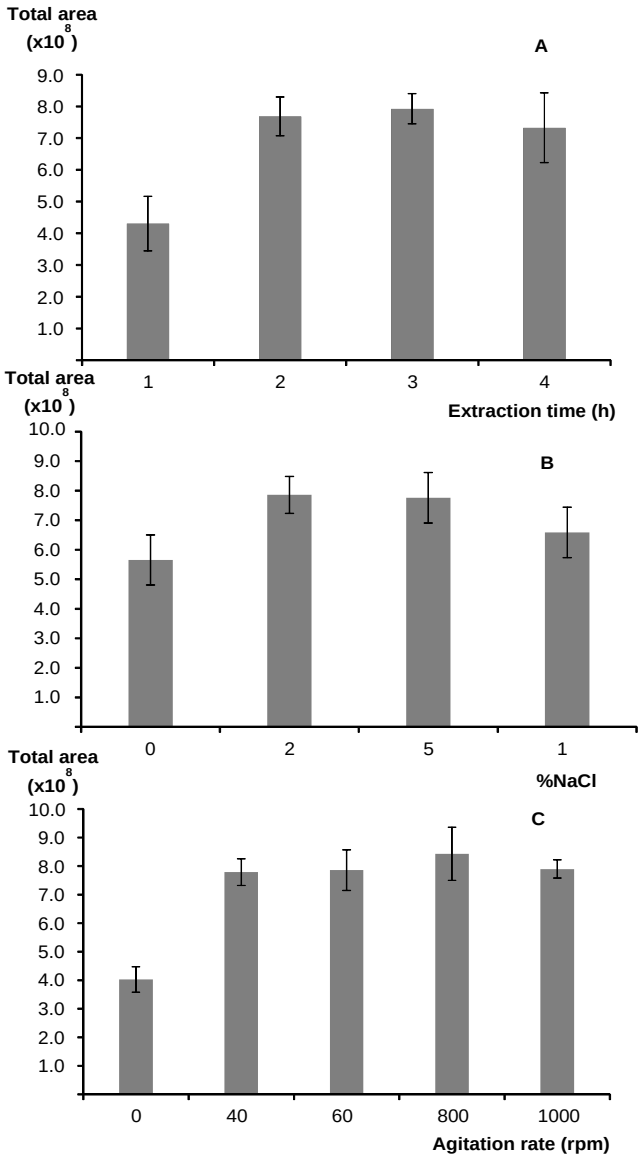

Figure 1: Effect of extraction time (A), % of NaCl (B), and agitation rate (C) on the total area of detected compounds. Vertical segments correspond to standard deviation (n=3).

3.2. SBSE-TD-GC-MS peach juice profiling

Using the optimized conditions described above, samples were analyzed by SBSE-TD-GC-MS. The areas achieved with the extracted ion of each of the identified compounds (Table 1) were normalized using 1-octanol as internal standard before proceeding with further chemometric treatment. The chromatograms showed several classes of chemical compounds, including the following: lactones typical of peach aroma, such as γ-hexalactone, γ-octalactone, γ-decalactone, δ-decalactone, γ-dodecalactone and δ-dodecalactone [5]; fatty acids (nonanoic, tridecanoic, hexadecanoic, and unsaturated C18 acids), which are precursors of aroma compounds such as lactones and esters [27]; fatty aldehydes (docosanal, tetracosanal, pentacosanal, hexacosanal and octacosanal); and linear alkanes (from C22 to C33). Long-chain aldehydes and hydrocarbons are typical components of epidermic wax. Other chemical classes, such as alcohols (nerol, 3-octen-2-ol, phytol, and α-tocopherol) and terpenoids (geranyl acetone), were also detected but in a lower abundance. In contrast, volatile compounds with linear retention indexes below 950 were not detected. These results are explained by the fact that it is not possible with the Peltier unit to lower the temperature below *ca.* 15 ºC. As expected, the sampling method by immersion of the stir bar allowed the detection of compounds other than the volatiles typically analyzed using the conventional approach of HS-SPME. For this reason, it would be feasible to classify distinct peach juice samples on the basis of the volatile and semi-volatile compounds present in the samples.

A typical GC-MS total ion count chromatogram (TIC) for peach juice is shown in Figure 2. The number of compounds refers to the identities of some components listed in Table 1 (minor compounds not labelled).

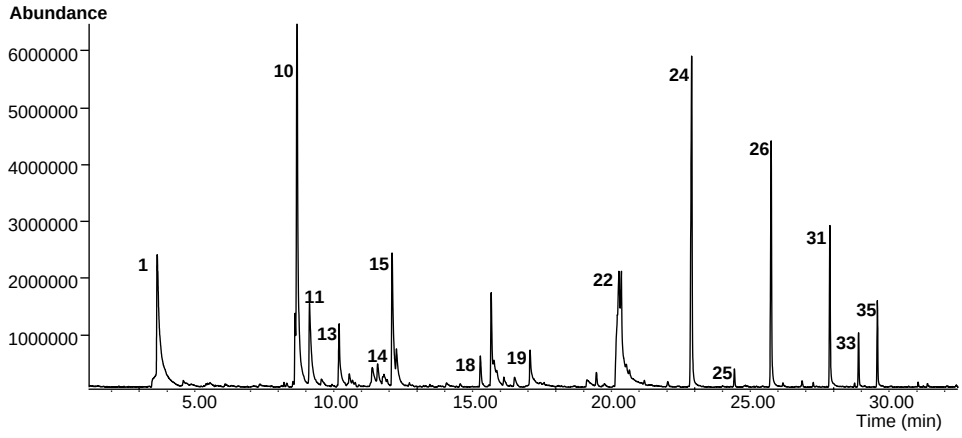

Figure 2: TIC of a peach juice sample (compounds are numbered according to Table 1).

3.3. Statistical analysis

Exploratory data analysis using PCA

A preliminary PCA study (Figure 3) was conducted using the SBSE-TD-GC-MS profile to evaluate the influence of peach variety and processing on the natural grouping of the samples on the basis of their metabolomic profiles. PC 1 and PC 2 captured 89.7% of the variance, while 95.5% of the total variance was explained when PC 3 was also considered. The score plots show that differentiation between yellow- and red-fleshed peach was achieved in PC 1. However, grouping of the samples was clearly observed only when considering yellow- and red-fleshed peach varieties. The analysis of loading vectors for PC 1 (Table 2) shows that several classes of compounds, such as lactones (γ-hexalactone, δ-decalactone, (*Z*)-6-γ-dodecenolactone and δ-dodecalactone), fatty aldehydes (docosanal and pentacosanal), fatty acids (tridecanoic and hexadecanoic), and hydrocarbons (C19, C21, C28, and C30), have strong positive contributions, i.e. increase in yellow-fleshed peach, while only a few have negative contributions, such as γ-decalactone, α-tocopherol and C33, i.e. increase in red-fleshed peach. The presence of lactones, especially γ-decalactone and δ-decalactone, as character impact in the differentiation of peach varieties on the basis of aroma has been reported [18]. Regarding long-chain compounds, such as fatty acids, fatty aldehydes and hydrocarbons, they do not contribute to the aroma of the juices; however, these compounds have been reported to be key components of the cuticle of the fruit [28], with a content that might differ between cultivars, as described by Belge et al. [29].

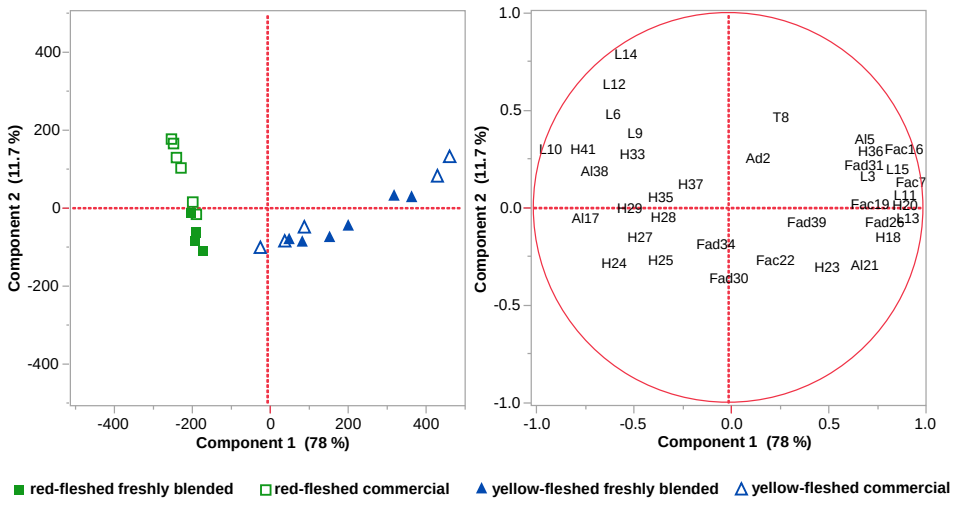

Figure 3: Score and loading plots obtained from the PCA analysis.

Table 2: Higher loading vector values obtained by PC 1.

Compounds	Code	Loadings on PC1
γ-hexalactone	L3	0.841
nonanoic acid	Fac7	0.877
γ-decalactone	L10	-0.841
δ-decalactone	L11	0.973
(*Z*)-6-γ-dodecenolactone	L13	0.897
δ-dodecalactone	L15	0.988
tridecanoic acid	Fac16	0.894
C19	H18	0.729
hexadecanoic acid	Fac19	0.891
C21	H20	0.792
docosanal	Fad26	0.792
pentacosanal	Fad31	0.810
C28	H32	0.784
C30	H36	0.810
α-tocopherol	Al38	-0.786
C33	H41	-0.843

For commercial and freshly blended samples, a slight tendency towards the differentiation of red-fleshed peaches was observed. In contrast, in the case of yellow-fleshed peaches, samples appeared to be scattered along the axis defined by PC 1. In addition, it should be noted that the PC 3 axis did not contribute to any further separation of the four types of samples (data not shown).

Discrimination of peach variety and production process using SLDA

Preliminary exploratory analysis of the profiles obtained by SBSE-TD-GC-MS using PCA revealed its potential use for classifying peach juices on the basis of variety. An SLDA algorithm was subsequently used to build a model able to discriminate between the two processing procedures to which the peaches were submitted, in addition to their variety. It is worth noting that while PCA selects a direction that retains maximum structure of data in a reduced dimension, LDA selects a direction that achieves maximum separation between given sample classes [30]. The SLDA model was constructed by applying a stepwise variable selection in which the most significant variables were chosen on the basis of their F-ratio value—higher F-ratio values maximize discrimination between

groups (Table 3). This strategy to perform the discriminant analysis led to a considerable reduction of the dimensionality of the information, resulting in the selection of only 14 variables considered to be those most important for the differentiation of the four groups of samples. The compounds selected for the SLDA model included lactones (6-pentyl-alpha-pyrone, γ-decalactone, γ-dodecalactone and δ-dodecalactone), fatty acids (hexadecanoic acid), fatty aldehydes (tetracosanal and octacosanal), hydrocarbons (C23, C26, C27, C29, and C33), and alcohols (phytol and α-tocopherol). The use of the 14 compounds resulted in 100% accurate classification of the four peach juice groups on the basis of variety and processing conditions. Figure 4 shows a plot obtained with the current classification model with the first two canonical variables. In canonical variable 1, a clear separation of samples on the basis of variety can be observed. Canonical variable 2 separates on the basis of processing conditions (freshly blended vs. commercial). In addition, to validate the model, samples not used for its construction were cross-validated, obtaining a success rate of 100%. Considering the loadings of the compounds used in the model, the rays show how each covariate loads onto the first two standardized canonical variables. The direction of a ray indicates the degree of association of that covariate with the first two canonical variables. Considering these points, canonical variable 1 is highly associated with fatty aldehydes (tetracosanal and octacosanal) and to a lesser extent with C23 and C26 hydrocarbons. In addition, other compounds are also weakly associated with canonical 1. Regarding canonical variable 2, it is highly associated with octacosanal, C23, hexadecanoic acid and α-tocopherol and to a lesser extent with 6-pentyl-α-pyrone and δ-dodecalactone.

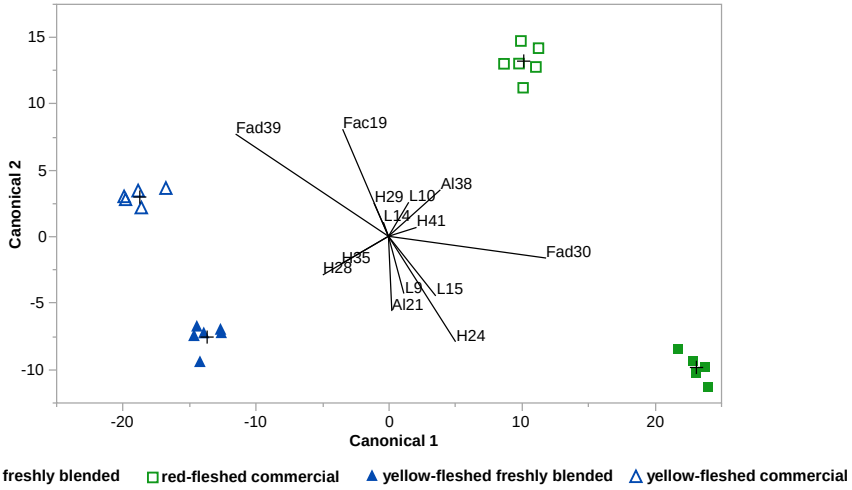

Figure 4: Plot of the two main canonical variables after SLDA and bi-plot rays representing the loadings of each selected compound.

Table 3: Most discriminating variables using a stepwise selection on the basis of the F-ratio.

Compound	F-Ratio[a]	Prob >F[b]
L9	5.35	0.05
L10	1.77	0.27
L14	0.52	0.69
L15	4.91	0.06
Fac19	5.76	0.04
Al21	17.36	0.00
H24	5.35	0.05
H28	2.07	0.22
H29	0.86	0.52
Fad30	5.24	0.05
H35	1.89	0.25
Al38	4.89	0.06
Fad39	18.75	0.00
H41	2.51	0.17

[a] F-ratio for a test for the group variable obtained using an analysis of covariance model.

[b] Prob >F: p-value for a test for the group variable obtained using an analysis of covariance model.

In summary, the proposed methodology is a useful tool to classify peach juice samples on the basis of agronomic (variety) and technological (processing) variables. The methodology is based on the following two steps: a) metabolomics profiling of the juices by SBSE-TD-GC-MS; and b) use of common chemometric tools to analyze these profiles. In this study, we have shown that, in addition to volatile compounds, which are usually analyzed by HS, fruit juices contain other semi-volatile compounds that can be useful for sample differentiation purposes. These compounds can be extracted from the liquid matrix by SBSE using a PDMS-coated stir bar. Furthermore, due to the nature of the extraction technique applied, SBSE can also be implemented for classification purposes in other liquid samples, such as other fruit juices or beverages.

Acknowledgments

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CHAPTER 4. GENERAL DISCUSSION

As stated in Chapter 2, the objectives of this Doctoral Thesis were to develop new analytical methods and applications in the field of metabolomics analysis in agrifood products. In this regard, we focused the scope of this Thesis on gas chromatography-mass spectrometry methods. The experimental section was divided in three sections considering the analytical approaches followed. Considering the compounds analysed and the techniques employed, the first and second sections were devoted to high-boiling compounds, which require a derivatization step prior to their analysis by GC. The derivatization reagent showing better results was MSTFA, which was combined in some cases with pyridine acting simultaneously as solvent and catalyst (Section 3.1.1. and 3.2.1.) or only as catalyst (Section 3.2.3). The last section of this Thesis was focused on the analysis of volatile and semi-volatile compounds. Due to their chemical structure, this group of compounds can be directly analysed by gas chromatography, thus avoiding the use of derivatization steps.

In the first section, GCxGC was employed to successfully detect and separate the disaccharides present in two different fruits, namely apple and peach. Disaccharides are compounds formed by two monosaccharide units, which provide them very similar structures difficult to separate employing conventional monodimensional GC systems [1]. GCxGC as a multidimensional technique enhances separation power and lowers detection limits [2]. In this study several column configurations were tested in the 2D system. The “classical” combination consisting on a non-polar/semi-polar setting did not provide a good spatial occupation of the second dimension. The polar/non-polar configuration conducted to the overlap with sucrose of peaks with lower retention time. Finally, the semi-polar/non-polar configuration allowed the optimum occupation of the 2D system. This allowed the successful analysis of the disaccharide composition of apple and peach. Moreover, the results revealed that composition between both fruits was statistically different. On the contrary, no statistical differences were found when analysing cultivars of the same fruit.

The second section of this Doctoral Thesis was focused on developing analytical methods based on injection-port derivatization to analyse several groups of compounds, which are of high interest in fruit and fruit juice production. The three research papers presented here include a step in which injection-port derivatization is optimized. Several parameters were considered such as derivatization reagent, purge-off time, injection-port temperature and ratio between sample and derivatization reagent as previously performed in other studies [3-5]. We observed that MSTFA was the most suitable reagent in most cases because it provided higher response in a given concentration. In addition, we observed that, when possible, it is better to select low purge-off times to avoid accumulation of dirt in the injection liner. Besides, high injection-port temperatures (270 and 280 ºC) were more suitable to achieve good performance in Section 3.2.2 and 3.2.3. However, such high temperatures were not found suitable when analysing glycosylated polyphenols as we detected a breakage of the glycosidic bond between the flavonoid and the sugar moiety. To avoid this problem we used a temperature ramp from 100 to 300 ºC in the injection-port.

The first research paper of Section 3.2 was devoted to the analysis of glycosylated and non-glycosylated polyphenols. Some attempts for the analysis of flavonoids by GC had already been performed [6]. However, MRM was not employed and thus limits of detection were not low enough in most cases for quantitative purposes. The developed method is an alternative to current methods, which are mostly based on liquid chromatography [7, 8]. However, when employing GC the analysis of these compounds in fruit samples requires a different sample treatment, i.e. clean-up of the aqueous-methanol extract employing a C18 SPE cartridge to remove sugars and organic acids. These compounds are present in samples in high abundance and compete with the target compounds leading to matrix effects in the injection liner at the in-port derivatization step. In addition, the organic extract is evaporated under vacuum and subsequently methoximated after clean-up. As mentioned above, MRM acquisition mode was employed in this part to achieve a good analytical performance given that in addition to lowering LOD. Indeed, MRM is a more selective mode and ideal for the target analysis and quantification of compounds for which we already have analytical standards.

The second study employing injection-port derivatization was focused on the development of a method to analyse HMF and patulin in fruit juices (mostly apple and pear). These two compounds are of high importance in fruit juice industry as can be considered markers of the quality of a fruit-derived product [9]. In this case, extraction with ethyl acetate was carried out as previously reported [10] giving good recoveries. Ethyl acetate is a solvent compatible with MSTFA since it does not contain hydroxyl groups. Moreover, no matrix effects were observed in this method. This allowed the direct introduction of the extract to the injection-port derivatization system. With this procedure, analysis time and reagent consumption were considerably reduced making this method a good alternative for current routine analysis methods in fruit juice industry. At the sample analysis step, several apple and pear juices were analysed to quantify the content of these two compounds.

In the last study of this section, we focused on the analysis of the free lipophilic fraction in fruit juices. Here, a liquid microextraction technique was optimized to extract the target compounds from the matrix in addition to injection-port derivatization. DLLME is a technique usually employed in aqueous matrices [11, 12]. Hence, a previous centrifugation and dilution was required to obtain matrices more similar to water since juice samples contain a considerable amounts of suspending solids, especially the samples with a high content of fruit pulp. Besides, a full factorial experimental design was conducted to achieve the conditions that maximize extraction. The most relevant parameters considered were extraction solvent, extraction solvent volume, and dispersion solvent. The extraction solvents in this technique need to be denser than water and immiscible, thus chlorinated solvents such as chloroform and chlorobenzene fulfil this characteristics. As dispersion solvent, typically acetone and methanol are selected because they need to be soluble both with the extraction solvent and the aqueous phase. Considering the extraction solvent, chloroform gave the best results. On the other hand, lower volumes led to higher EF as expected. However, it was difficult to collect, in some samples, such low volumes after centrifugation and the repeatability of the method was

not good enough. For this reason, we selected 100 µL of chloroform instead of 50 µL. Finally, considering the dispersion solvent, a similar phenomenon was observed. While methanol gave higher EF, an interphase was formed between the extraction solvent and the sample after centrifugation. Hence, acetone was selected as disperser.

Section 3.3. involved two research papers. The first of them was devoted to the analysis of volatile and semi-volatile compounds in fruit fibres deriving from juice industry by-products. Results achieved from the analysis by HS-SPME-GC-MS revealed that the presence of remaining volatiles is very low in non-citrus when compared with citrus fibres. Indeed, the volatiles composition suffered an important diminishment when compared with the corresponding fresh fruits [13-15]. A possible explanation to this phenomenon is that the volatile composition of raw apple, pear, and peach has a predominance of more volatile compounds such as short-chain aldehydes and esters, which are more prone to be removed during the drying process applied to the by-products. On the other hand, citrus fibres kept a composition much more similar to that found in raw citrus fruits. This composition is clearly dominated by terpenoids, being limonene the most abundant among them. Moreover, the volatile content found in orange and tangerine peel was higher than that of the corresponding fleshes as happens with raw fruits [16, 17]. The second part of this research paper was devoted to the enantioselective analysis of the chiral terpenoids present in the samples. We found that monoterpene alcohols, (terpinen-4-ol and α-terpineol) tend to show a variation in their ER, probably because of the heat applied during drying. This observation was in agreement with the previously reported by Dugo and Mondelo when comparing the enantiomeric ratio of citrus oil obtained by cold-pressing or hydrodistillation [18].

The second study of Section 3.3. was focused on the development of a SBSE-TDU coupled with GC-MS. Conventional chemometric techniques were applied to distinguish between peach juices of distinct varieties, namely yellow- and red-fleshed, and between two processing conditions —commercial and freshly blended. Traditionally, these analyses have been carried out by sampling the HS with SPME [19-22] due to the possible degradation of the fibre when working in DI. Besides, SBSE was initially conceived for its use in DI mode and showed to be a useful approach to extract an important number of volatile and semi-volatile compounds present in peach juice samples as reported in the study. The data obtained was treated using chemometric techniques. Initially, PCA allowed the distinction between the two varieties but not between the processing conditions. Hence, a SLDA allowed the exclusion of some of the variables that introduced noise to the model and showed that the combination of 14 variables allowed the distinction between processing in addition to variety. The model was cross-validated introducing samples that were not considered in the construction of the model confirming its success. Among the compounds used in the SLDA model there are lactones, fatty acids, fatty aldehydes, hydrocarbons, and alcohols.

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CHAPTER 5. GENERAL CONCLUSIONS

After presenting and discussing the results achieved in this Thesis, the main conclusions are exposed in this Chapter as follows:

1. GC×GC-MS has proven to be very useful to study the complexity of disaccharide analysis in fruit samples, especially when using a semi-polar/non-polar configuration. In addition, focussing of the analytes caused by the cryomodulator allows the achievement of lower detection limits. Hence, increasing detectability of minor compounds

2. When applying the developed GC×GC-MS method to peach an apple samples, results revealed compositional differences between fruits. However, cultivars within fruits did not show statistical differences when comparing their disaccharide content.

3. Injection-port derivatization is an effective technique to silylate compounds prior to their analysis by GC-MS. This technique reduced analysis time and silylation reagent consumption besides increasing automation of the analysis and thus improving repeatability.

4. The high content of sugars and organic acids in the fruit samples led to important matrix effects if polyphenols were analysed without a previous clean-up. The introduction of a previous step based on the use of an SPE cartridge containing C18 phase allowed the analysis of glycosylated and non-glycosylated polyphenols. The developed methodology allowed a fast target analysis of polyphenols by GC, which represents an alternative to conventional HPLC methods.

5. Given an extraction solvent compatible with direct silylation, i.e. a solvent not containing free -OH groups, injection-port derivatization can be easily performed as shown in the method developed for the analysis of two important compounds in fruit juice industry (HMF and patulin). If no matrix effects were observed, direct analysis of the extract would be preferred to achieve lower LOD and LOQ.

6. A microextraction method by DLLME was developed to analyse the free lipophilic fraction in fruit juice samples. In this regard, full factorial experimental design showed to be a good option to maximize enrichment factors and find out which are the parameters with a higher influence.

7. Employing 100 µL $CHCl_3$ as extraction solvent and 1 mL acetone as disperser a fast and efficient extraction of fatty acids, fatty alcohols, phytosterols, and triterpenes was performed. The resulting extract can be directly analysed by GC-MS giving a remarkable performance.

8. For the analysis of volatile and semi-volatile compounds in fruit-derived samples the use of organic solvents can be avoided. In this regard, solid-phase microextraction techniques such as SPME and SBSE play an important role.

9. SPME, as a robust and widely applied technique, allowed the characterisation of the volatile fraction of several types of fruit fibres from the juices industry. As SPME can be automated, this is the appropriate technique when a high number samples has to be analysed.

10. Enantiomeric analysis of asymmetric terpenoids revealed that terpinen-4-ol and α-terpineol showed variation on the ER, probably due to the drying process of the fruit fibres.

11. For direct immersion analysis SBSE employing a PDMS-coated stir bar is a more suitable technique than SPME. This might suffer degradation of the phase after a few analyses. In addition, thermal desorption can also be performed employing a thermal desorption unit.

12. Conventional chemometric tools applied to SBSE-GC-MS analytical data of peach juices from peach varieties and different processing conditions revealed that 14 variables were necessary to distinguish between the four groups of samples.