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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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HIGHLIGHTS

- The method allows the determination of free lipophilic fraction in juices.
- DLLME has been optimized using full factorial experimental design.
- Injection-port derivatization with dual injection of the extract is performed.
- DLLME combined with in-port derivatization reduces reagents consumption and analysis time.

Abstract

A method consisting of dispersive liquid-liquid microextraction (DLLME) followed by injection-port derivatization and gas chromatography-mass spectrometry (GC-MS) 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 *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA):pyridine (1:1) as reagent. Quality parameters were assessed for the target compounds, giving a limits of detection (LODs) ranging from 1.1 to 5.7 ng/mL and limits of quantification (LOQs) 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 $r^2 \geq 0.990$ for all ranges applied. Finally, the method was used to test 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 edaphoclimatic [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. The method consist on coupling a microextraction technique with an injection-port derivatization, which represents an innovative approach to analyze lipophilic compounds in liquid samples 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.

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, 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 electron ionization (EI) ion source. The mass spectrometer worked in selected ion monitoring (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 and diluted 1:10 with distilled water.Subsequently, a 5.0-mL aliquot of diluted juice was transferred to 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, i.e. 1 µL of derivatization reagent and 1 µL of sample in a 10 µL syringe, 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 polytetrafluoroethylene (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 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. Blank samples were analyzed during the sample sequence to check the absence of carry-over effects.

2.4. Statistical analysis

Full factorial experimental designs and mean chart plots were carried out with JMP 12.0.1 from SAS Institute Inc. (Cary, NC, USA). Analysis of variance (ANOVA) was performed with Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA).

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 to reduce the risk of dirtiness accumulation in the injection liner when using high purge-off times. 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) consisting of 1 µl of both sample and derivatization reagent was selected.

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).

3.3. 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 these matrices. 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 conducted 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 the analytes. Recoveries were calculated with the following equation, where C_{real} refers to the concentration of a given compound naturally present in the sample (1):

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

We observed that an interphase was formed 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 considered acceptable for all compounds (Table 3), 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 using 100 µL of $CHCl_3$ as the best conditions to perform the lipophilic fraction analysis of the juice samples.

3.4. Method performance

The performance parameters of the GC-MS method for the optimized conditions were assessed in terms of limits of detection (LODs), limits of quantification (LOQs), coefficient of determination (r^2), linear range (by plotting peak area against concentration), and repeatability according to ICH Harmonised Tripartite Guideline [28], as summarized in Table 4. A synthetic juice was used for this purpose. LODs and LOQs 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 LODs obtained were low, ranging from 1.1 to 5.7 ng/mL while the LOQs 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 %of relative standard deviation (%RSD), n=5) achieved values below 11.51%. Finally, no carry over effects were observed when analyzing a blank sample of synthetic juice at the end of a sample sequence.

The proposed method was compared with others reported in the literature, which employ GC-MS for the analysis of lipophilic compounds in vegetable matrices (Table 5). As mentioned before, there are no methods including the analysis of fatty acids, fatty alcohols, phytosterols and triterpenes in a single step. Moreover, microextraction techniques are very rarely used for this sort of chemicals, only the analysis of phytosterols in juices by SPME is reported [29] and the coupling with injection-port has

not been reported for these samples. In addition, the proposed method is comparable with or even better than the reported techniques in terms of performance parameters. Although it is difficult to compare between methods, because none of the reported methods include such a broad spectrum of lipophilic compounds families, it can be stated that LODs are better than the ones obtained by liquid extraction [25, 30, 31] and DI-SPME with on-fibre derivatization [29] and slightly higher than the ones obtained by SPE for fatty acids [32]. In addition, the method proposed has the advantage of reducing in a great extent the use of solvents and derivatization reagent together with an important saving of sample preparation time due to the automation achieved with injection-port derivatization.

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 6 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 when compared with a 100% fruit juice. The behavior of linoleic acid content differed in a wide range, 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 890.3 ng/mL in apple and carrot juice, 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 [33], and in pear, where palmitic acid content was higher than oleic acid [34]. 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 [35]. In addition, hexacosanol and octacosanol have been reported as the main fatty alcohols in several species of berries [36]. 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 (1,801.6 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 Where β-sitosterol is reported to be the main phytosterol in orange [8, 29], pineapple, berries [8] , mango, and apple juice [37], followed in all cases by campesterol [38]. Finally, triterpenes were found in only some of the samples, namely apple, pear, and peachIn this regard, α-amyrin was only detected in measurable amounts in apple juice while the contents of oleanloic and ursolic acid ranged from 190.2 to 267.2 ng/mL and from 261.5 to 848.9 ng/mL, respectively. These results are in agreement with previous research reporting the presence of oleanolic and ursolic acids in apple skin [39] and pear fruit [40].

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 LODs, 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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References

[1] Â.C. Salvador, S.M. Rocha, A.J.D. Silvestre, Lipophilic phytochemicals from elderberries (Sambucus nigra L.): Influence of ripening, cultivar and season, Ind. Crop. Prod., 71 (2015) 15-23.

[2] M. Teleszko, A. Wojdyło, M. Rudzińska, J. Oszmiański, T. Golis, Analysis of Lipophilic and Hydrophilic Bioactive Compounds Content in Sea Buckthorn (Hippophaë rhamnoides L.) Berries, J. Agric. Food Chem., 63 (2015) 4120-4129.

[3] S.A.O. Santos, C. Vilela, J.F. Camacho, N. Cordeiro, M. Gouveia, C.S.R. Freire, A.J.D. Silvestre, Profiling of lipophilic and phenolic phytochemicals of four cultivars from cherimoya (Annona cherimola Mill.), Food Chem., 211 (2016) 845-852.

[4] R. Domingues, G. Sousa, C. Silva, C. Freire, A. Silvestre, C.P. Neto, High value triterpenic compounds from the outer barks of several Eucalyptus species cultivated in Brazil and in Portugal, Ind. Crop. Prod., 33 (2011) 158-164.

[5] B.V. Howard, D. Kritchevsky, Phytochemicals and Cardiovascular Disease A Statement for Healthcare Professionals From the American Heart Association, Circulation, 95 (1997) 2591-2593.

[6] L. Normen, S. Bryngelsson, M. Johnsson, P. Evheden, L. Ellegård, H. Brants, H. Andersson, P. Dutta, The phytosterol content of some cereal foods commonly consumed in Sweden and in the Netherlands, J. Food Compos. Anal., 15 (2002) 693-704.

[7] T. Vanmierlo, C. Husche, H.F. Schött, H. Pettersson, D. Lütjohann, Plant sterol oxidation products – Analogs to cholesterol oxidation products from plant origin?, Biochimie, 95 (2013) 464-472.

[8] V. Piironen, J. Toivo, R. Puupponen-Pimiä, A.M. Lampi, Plant sterols in vegetables, fruits and berries, J. Sci. Food Agric., 83 (2003) 330-337.

[9] L.I. Somova, F.O. Shode, P. Ramnanan, A. Nadar, Antihypertensive, antiatherosclerotic and antioxidant activity of triterpenoids isolated from Olea europaea, subspecies africana leaves, J. Ethnopharmacol., 84 (2003) 299-305.

[10] M. Rezaee, Y. Assadi, M.-R.M. Hosseini, E. Aghaee, F. Ahmadi, S. Berijani, Determination of organic compounds in water using dispersive liquid–liquid microextraction, J. Chromatogr. A, 1116 (2006) 1-9.

[11] K. Blau, G.S. King, Handbook of derivatives for chromatography, Wiley New York, 1993.

[12] V.G Berezkin, Chemical methods in gas chromatography, Elsevier, 2000.

[13] K.-L. Woo, J.-I. Kim, New hydrolysis method for extremely small amount of lipids and capillary gas chromatographic analysis as N(O)-tert.-butyldimethylsilyl fatty acid derivatives compared with methyl ester derivatives, J. Chromatogr. A, 862 (1999) 199-208.

[14] M. Pelillo, G. Galletti, G. Lercker, Mass spectral fragmentations of cholesterol acetate oxidation products, Rapid Commun. Mass Spectrom., 14 (2000) 1275-1279.

[15] P.C. Dutta, L. Normén, Capillary column gas–liquid chromatographic separation of Δ5-unsaturated and saturated phytosterols1, J. Chromatogr. A, 816 (1998) 177-184.

[16] D.-K. Nguyen, A. Bruchet, P. Arpino, Determination of sterols in sewage sludge by combined in situ trimethylsilylation/supercritical fluid extraction and GC/MS, Environ. Sci. Technol., 29 (1995) 1686-1690.

[17] A. Ghosh, S. Misra, A.K. Dutta, A. Choudhury, Pentacyclic triterpenoids and sterols from seven species of mangrove, Phytochemistry, 24 (1985) 1725-1727.

[18] M. Burnouf-Radosevich, N.E. Delfel, R. England, Gas chromatography-mass spectrometry of oleanane-and ursane-type triterpenes—application to Chenopodium quinoa triterpenes, Phytochemistry, 24 (1985) 2063-2066.

[19] K.S. Docherty, P.J. Ziemann, On-line, inlet-based trimethylsilyl derivatization for gas chromatography of mono- and dicarboxylic acids, J. Chromatogr. A, 921 (2001) 265-275.

[20] A. Marsol-Vall, M. Balcells, J. Eras, R. Canela-Garayoa, Injection-port derivatization coupled to GC–MS/MS for the analysis of glycosylated and non-glycosylated polyphenols in fruit samples, Food Chem., 204 (2016) 210-217.

[21] A. Marsol-Vall, M. Balcells, J. Eras, R. Canela-Garayoa, A rapid gas chromatographic injection-port derivatization method for the tandem mass spectrometric determination of patulin and 5-hydroxymethylfurfural in fruit juices, J. Chromatogr. A, 1453 (2016) 99-104.

[22] E. Bizkarguenaga, A. Iparragirre, P. Navarro, M. Olivares, A. Prieto, A. Vallejo, O. Zuloaga, In-port derivatization after sorptive extractions, J. Chromatogr. A, 1296 (2013) 36-46.

[23] P. Viñas, N. Martínez-Castillo, N. Campillo, M. Hernández-Córdoba, Directly suspended droplet microextraction with in injection-port derivatization coupled to gas chromatography–mass spectrometry for the analysis of polyphenols in herbal infusions, fruits and functional foods, J. Chromatogr. A, 1218 (2011) 639-646.

[24] H. Scherz, F. Senser, Food composition and nutrition tables, Medpharm GmbH Scientific Publishers, 1994.

[25] J. Wu, R. Hu, J. Yue, Z. Yang, L. Zhang, Determination of fecal sterols by gas chromatography-mass spectrometry with solid-phase extraction and injection-port derivatization, J. Chromatogr. A, 1216 (2009) 1053-1058.

[26] A. Zgoła-Grześkowiak, T. Grześkowiak, Dispersive liquid-liquid microextraction, Trends Anal. Chem., 30 (2011) 1382-1399.

[27] P. Viñas, N. Campillo, I. López-García, M. Hernández-Córdoba, Dispersive liquid–liquid microextraction in food analysis. A critical review, Anal. Bioanal. Chem., 406 (2014) 2067-2099.

[28] I.H.T. Guideline, Validation of analytical procedures: text and methodology, Q2 (R1), 1 (2005).

[29] S. Balme, F.O. Gülaçar, Rapid screening of phytosterols in orange juice by solid-phase microextraction on polyacrylate fibre derivatisation and gas chromatographic–mass spectrometric, Food Chem., 132 (2012) 613-618.

[30] A. Lytovchenko, R. Beleggia, N. Schauer, T. Isaacson, J.E. Leuendorf, H. Hellmann, J.K. Rose, A.R. Fernie, Application of GC-MS for the detection of lipophilic compounds in diverse plant tissues, Plant methods, 5 (2009) 4.

[31] S. Jäger, H. Trojan, T. Kopp, M.N. Laszczyk, A. Scheffler, Pentacyclic triterpene distribution in various plants–rich sources for a new group of multi-potent plant extracts, Molecules, 14 (2009) 2016-2031.

[32] B. Jurado-Sánchez, E. Ballesteros, M. Gallego, Gas chromatographic determination of 29 organic acids in foodstuffs after continuous solid-phase extraction, Talanta, 84 (2011) 924-930.

[33] J. Wu, H. Gao, L. Zhao, X. Liao, F. Chen, Z. Wang, X. Hu, Chemical compositional characterization of some apple cultivars, Food Chem., 103 (2007) 88-93.

[34] J. Chen, Z. Wang, J. Wu, Q. Wang, X. Hu, Chemical compositional characterization of eight pear cultivars grown in China, Food Chem., 104 (2007) 268-275.

[35] G. Verardo, E. Pagani, P. Geatti, P. Martinuzzi, A thorough study of the surface wax of apple fruits, Anal. Bioanal. Chem., 376 (2003) 659-667.

[36] L. Klavins, L. Klavina, A. Huna, M. Klavins, Polyphenols, carbohydrates and lipids in berries of Vaccinium species, Environ. Exp. Biol., 13 (2015) 147-158.

[37] A.I. Decloedt, A. Van Landschoot, L. Vanhaecke, Fractional factorial design-based optimisation and application of an extraction and UPLC-MS/MS detection method for the quantification of phytosterols in food, feed and beverages low in phytosterols, Anal. Bioanal. Chem., 408 (2016) 7731-7744.

[38] C.M. Martins, F.A. Fonseca, C.A. Ballus, A.M. Figueiredo-Neto, A.D. Meinhart, H.T. de Godoy, M.C. Izar, Common sources and composition of phytosterols and their

estimated intake by the population in the city of São Paulo, Brazil, Nutrition, 29 (2013) 865-871.

[39] C.M. Andre, J.M. Greenwood, E.G. Walker, M. Rassam, M. Sullivan, D.l. Evers, N.B. Perry, W.A. Laing, Anti-inflammatory procyanidins and triterpenes in 109 apple varieties, J. Agric. Food Chem., 60 (2012) 10546-10554.

[40] X. Li, T. Wang, B. Zhou, W. Gao, J. Cao, L. Huang, Chemical composition and antioxidant and anti-inflammatory potential of peels and flesh from 10 different pear varieties (Pyrus spp.), Food Chem., 152 (2014) 531-538.

[41] Z. Jemmali, A. Chartier, C. Dufresne, C. Elfakir, Optimization of the derivatization protocol of pentacyclic triterpenes prior to their gas chromatography–mass spectrometry analysis in plant extracts, Talanta, 147 (2016) 35-43.

Figure captions

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).

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

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

Figure 1

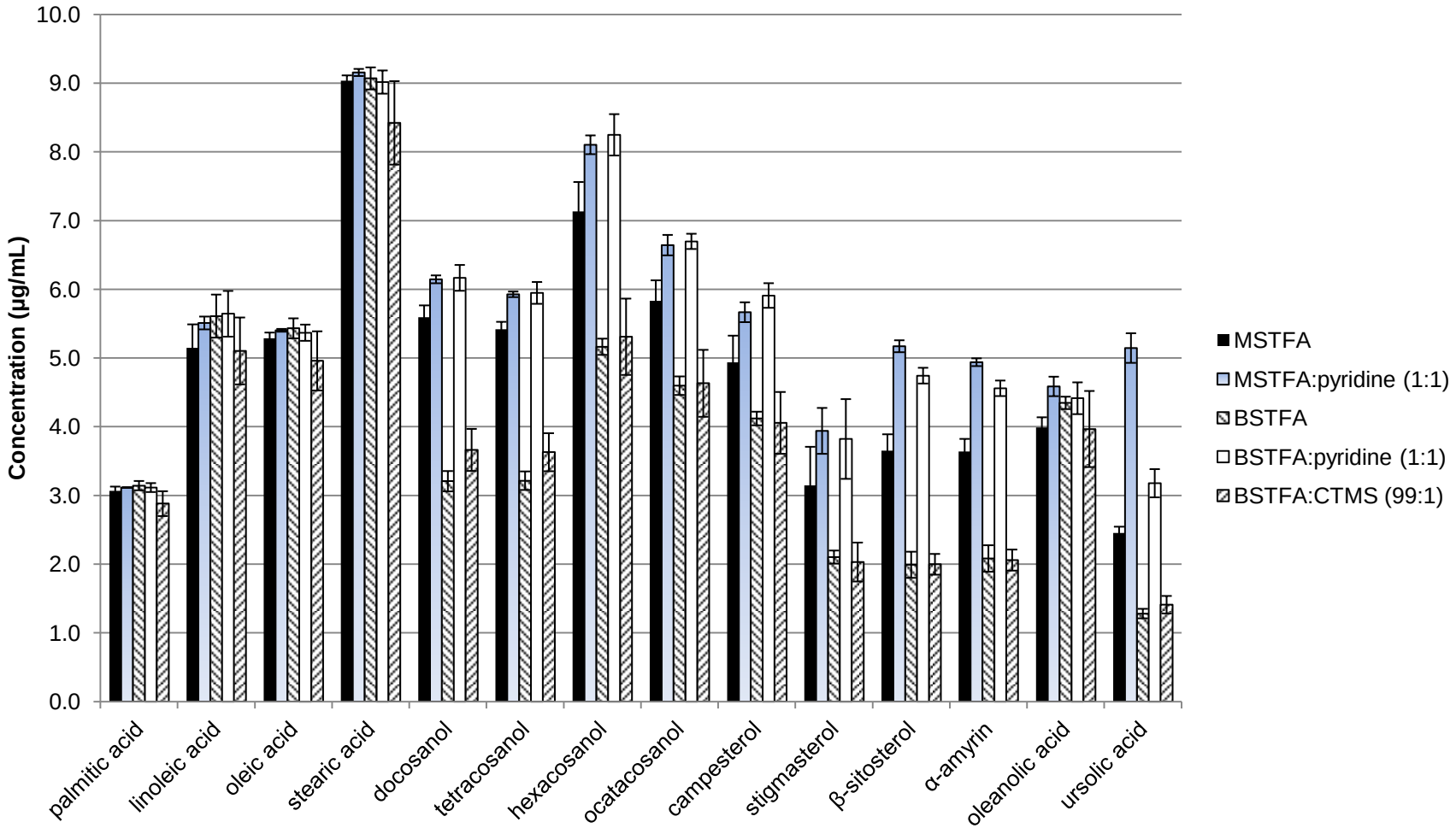

Concentration (µg/mL)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
palmitic acid
linoleic acid
oleic acid
stearic acid
docosanol
tetracosanol
hexacosanol
ocatacosanol
campesterol
stigmasterol
β-sitosterol
α-amyrin
oleanolic acid
ursolic acid
MSTFA
MSTFA:pyridine (1:1)
BSTFA
BSTFA:pyridine (1:1)
BSTFA:CTMS (99:1)

Figure 2

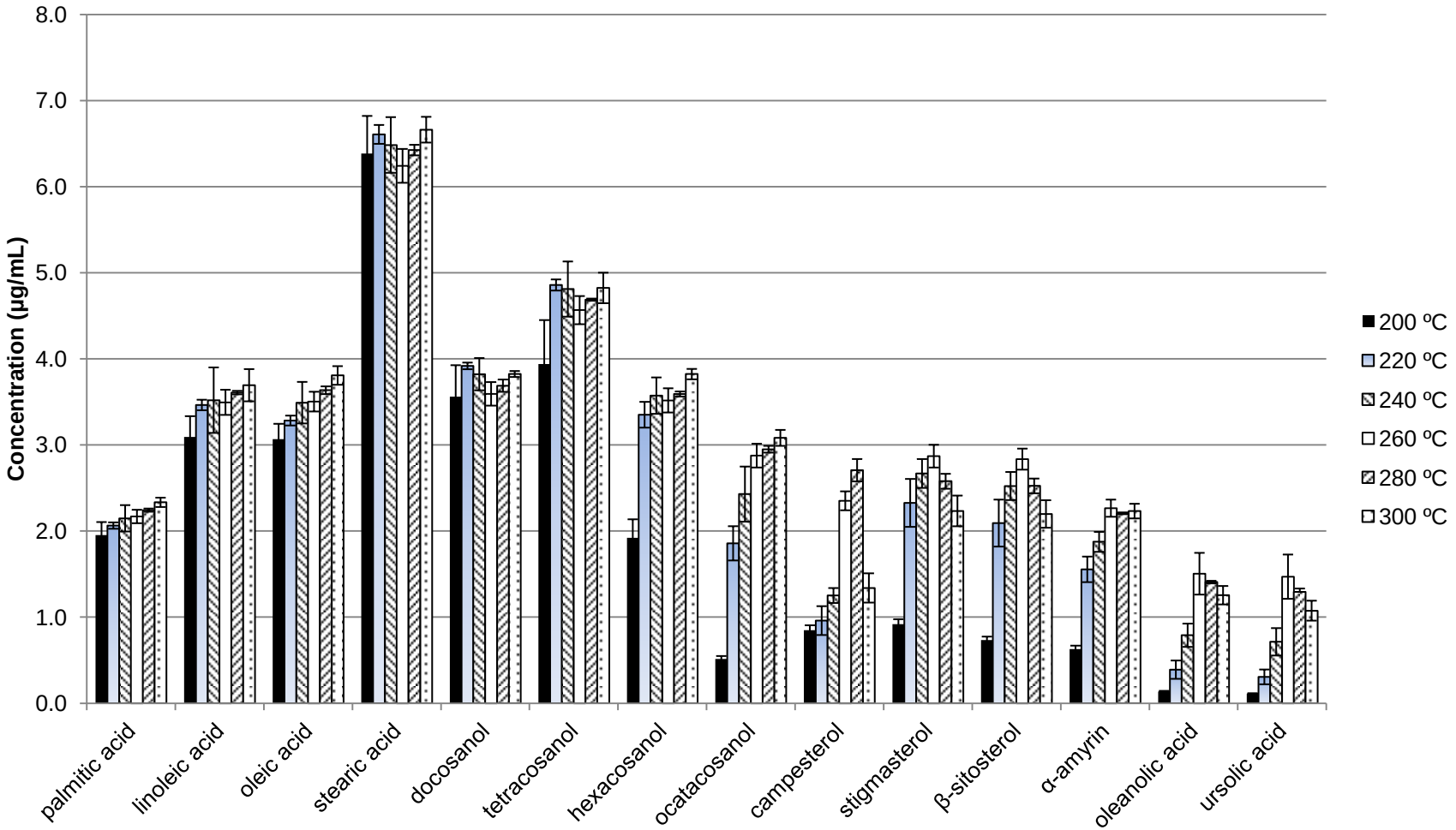

Concentration (µg/mL)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
palmitic acid
linoleic acid
oleic acid
stearic acid
docosanol
tetracosanol
hexacosanol
ocatacosanol
campesterol
stigmasterol
β-sitosterol
α-amyrin
oleanolic acid
ursolic acid
200 ºC
220 ºC
240 ºC
260 ºC
280 ºC
300 ºC

Figure 3

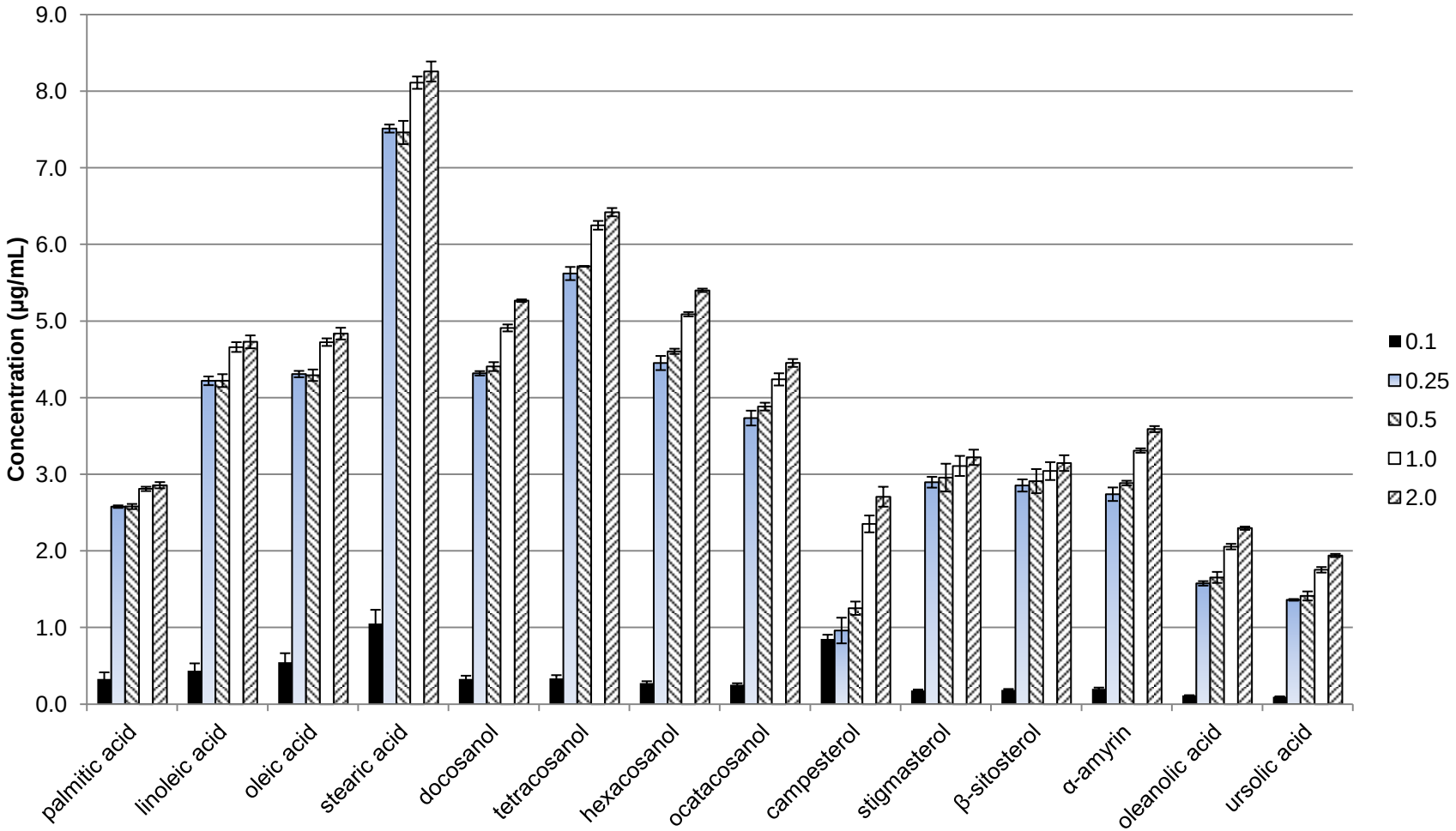

Concentration (µg/mL)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
palmitic acid
linoleic acid
oleic acid
stearic acid
docosanol
tetracosanol
hexacosanol
octacosanol
campesterol
stigmasterol
β-sitosterol
α-amyrin
oleanolic acid
ursolic acid
0.1
0.25
0.5
1.0
2.0

Figure 4

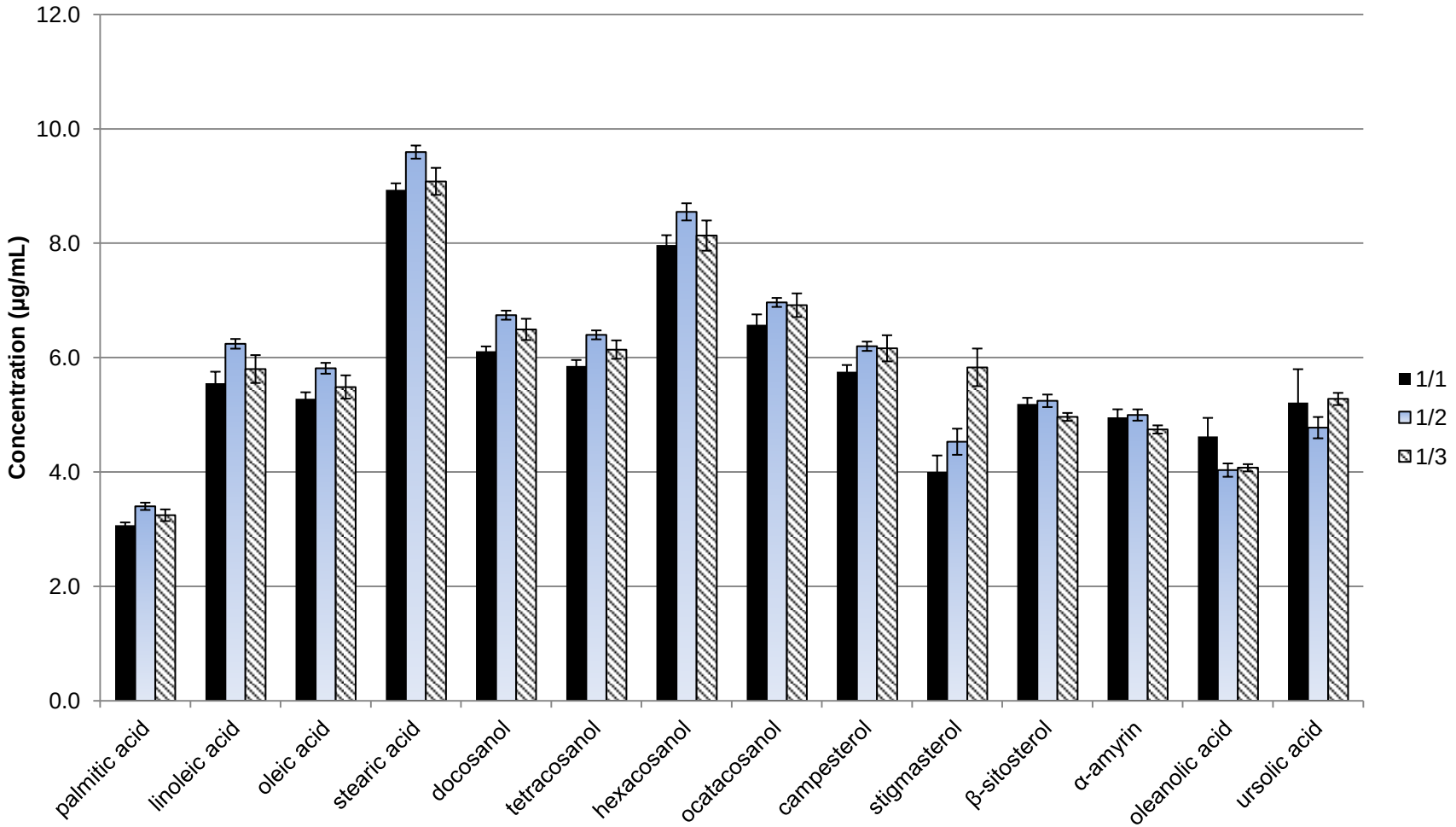
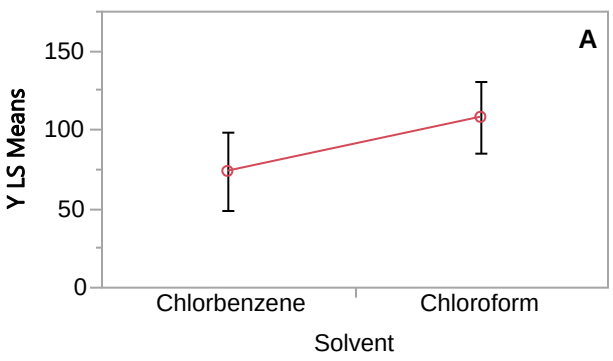
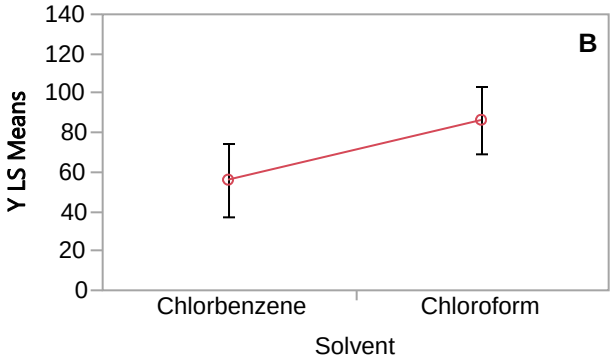
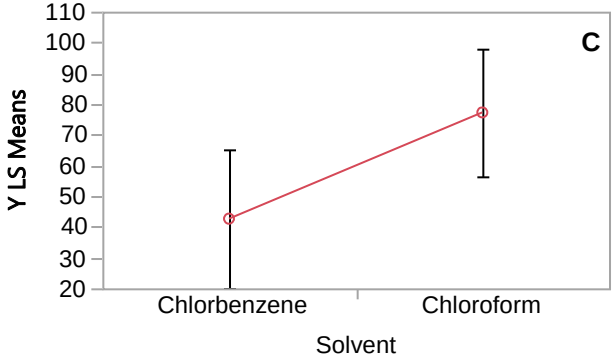
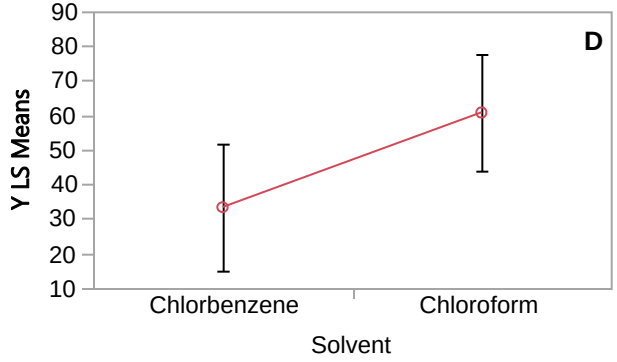

12.0
10.0
8.0
6.0
4.0
2.0
0.0
Concentration (µg/mL)
palmitic acid
linoleic acid
oleic acid
stearic acid
docosanol
tetracosanol
hexacosanol
ocatacosanol
campesterol
stigmasterol
β-sitosterol
α-amyrin
oleanolic acid
ursolic acid
1/1
1/2
1/3

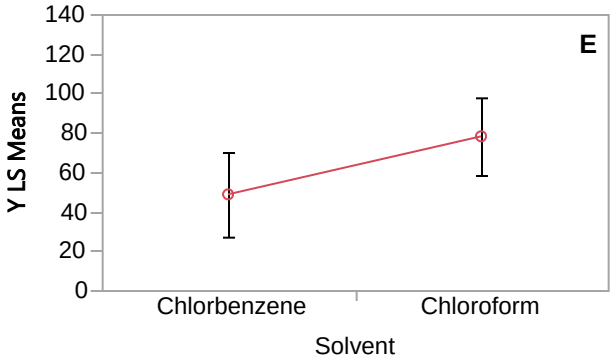
Figure 5

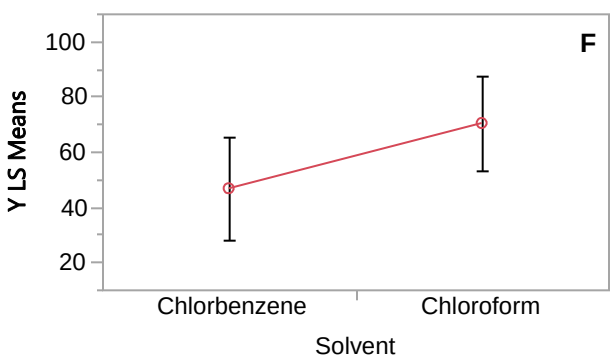

A
Y LS Means
150
100
50
0
Chlorbenzene
Chloroform
Solvent

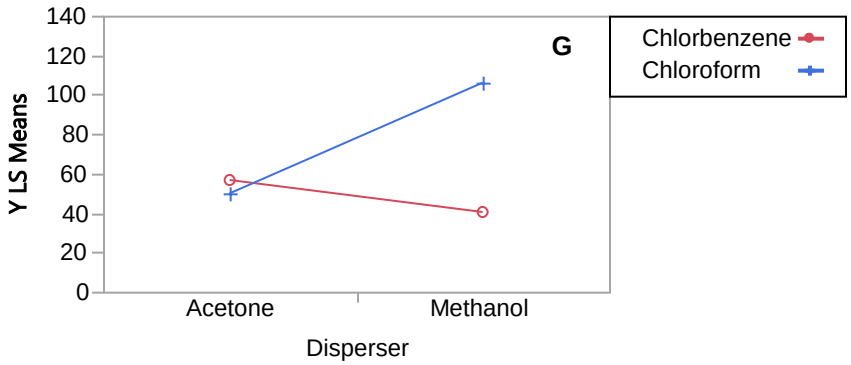

B
Y LS Means
140
120
100
80
60
40
20
0
Chlorbenzene
Chloroform
Solvent


C
Y LS Means
110
100
90
80
70
60
50
40
30
20
Chlorbenzene
Chloroform
Solvent


D
Y LS Means
90
80
70
60
50
40
30
20
10
Chlorbenzene
Chloroform
Solvent


E
Y LS Means
140
120
100
80
60
40
20
0
Chlorbenzene
Chloroform
Solvent


F
Y LS Means
100
80
60
40
20
Chlorbenzene
Chloroform
Solvent


G
Y LS Means
140
120
100
80
60
40
20
0
Acetone
Methanol
Disperser
Chlorbenzene
Chloroform

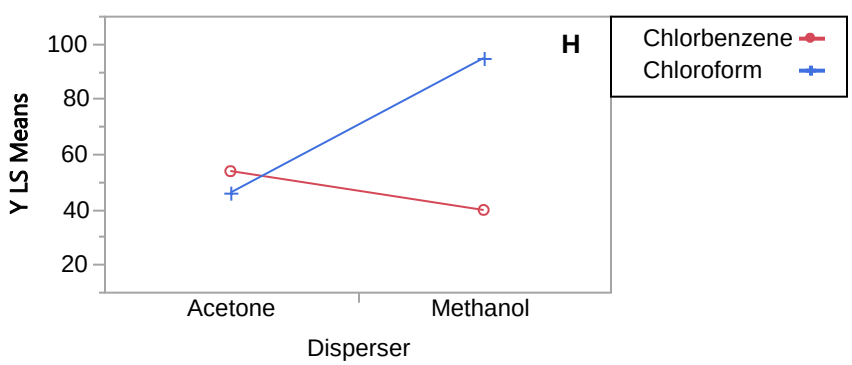

H
Y LS Means
100
80
60
40
20
Acetone
Methanol
Disperser
Chlorbenzene
Chloroform

Figure 6

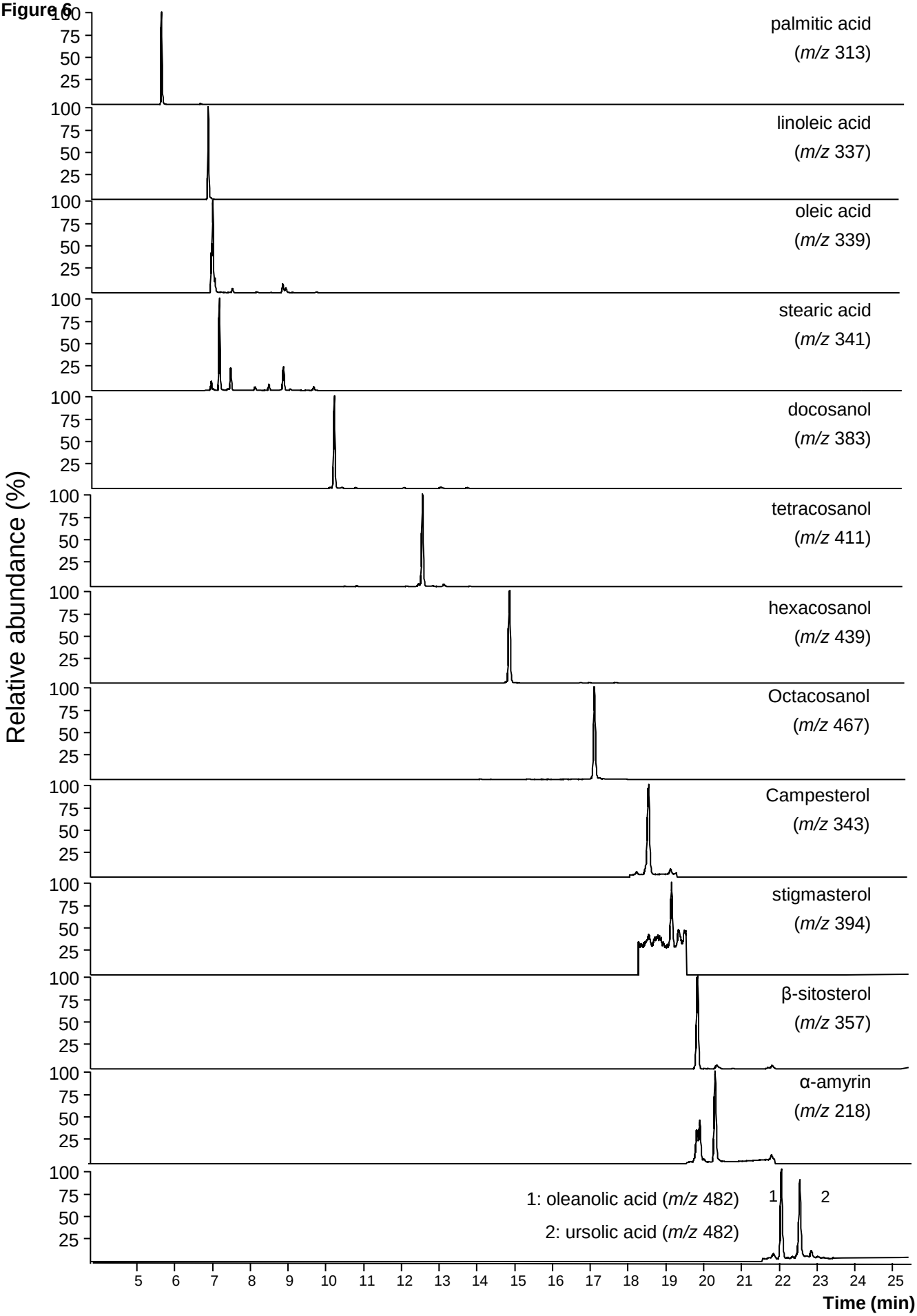

palmitic acid
(m/z 313)
linoleic acid
(m/z 337)
oleic acid
(m/z 339)
stearic acid
(m/z 341)
docosanol
(m/z 383)
tetracosanol
(m/z 411)
hexacosanol
(m/z 439)
Octacosanol
(m/z 467)
Campesterol
(m/z 343)
stigmasterol
(m/z 394)
β-sitosterol
(m/z 357)
α-amyrin
(m/z 218)
1: oleanolic acid (m/z 482)
2: ursolic acid (m/z 482)
1
2
Relative abundance (%)
100
75
50
25
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
Time (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.

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 volume	C: Disperser	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).

Table 3: Recoveries (%) and RSD (%, n=3) for berry and peach juice samples 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%)

Table 4: Performance parameters of the DLLME-GC-MS method in terms of LODs, LOQs, coefficient of determination (r^2), linear range, and repeatability.

Compound	LODs	LOQs	r^2	Linear range	Repeatability
	(ng/mL)	(ng/mL)		(ng/mL)	(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

Table 5: Comparison of the proposed DLLME-injection port-GC-MS with other methods for the analysis of the lipophilic fraction of vegetable samples.

Method[a]	LODs	LOQs	r^2	Linear range	Repeatability	Compounds analyzed				Ref.
	(ng/mL)	(ng/mL)		(ng/mL)	(RSD, %)	FA	FAL	PS	TRIT	
LE+drying	-	-	0.977-0.999	1.25-100 ng	6.8-15.5	x	x	x		[30]
LE+drying	300	-	0.990	3-50 mg/l	11				x	[41],
ASE+drying	0.03g/ 100g	0.10g/ 100g	-	-	5				x	[31]
DI-SPME	4.1	-	0.990	1.6-1600 mg/l	8			x		[29]
SPE (no derivatization)	0.4-0.8		-	1-1000	5.1-6.7	x				[32]
DLLME+in-port	1.1-5.7	3.4-18.7	0.990-0.999	10-1500	3.63-11.51	x	x	x	x	This work

[a] Derivatization was employed in all methods unless otherwise specified.

FA: fatty acids, FAL: fatty alcohols, PS: phytosterols, TRIT: triterpene

Table 6: DLLME-GC-MS analysis of commercial fruit juice samples (ng/mL).

Compounds	Apple juice		Apple concentrate juice		Pear juice		Mango juice		Peach juice		Orange juice		Apple and carrot juice		Pineapple juice		Berries juice	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Palmitic acid	865	750.2	n.d.	n.d	754.9	690	956	901.4	743.6	540.3	837.2	976.8	819.2	890.3	673.9	720.9	436	520.4
Linoleic acid	4087.9	3950.1	11.4	15.6	3142	2900.7	620.5	636.8	2477.4	2104.9	3098.5	4300	4244.7	3943.8	1123.6	1953.8	1350.1	1480.7
Oleic acid	609.6	435.5	n.d.	n.d.	573.7	525.4	889.2	854.5	221	260.8	616.3	849.6	569.1	890.3	876.2	540.2	669.1	430.3
Stearic acid	369.1	227.2	n.d.	n.d.	282.6	210.3	116.7	180.5	173.5	102.1	87.4	168.1	139.5	132.7	70	95.3	86.2	151.1
Docosanol	21.4	10.3	<LOQ	n.d	215.9	184.1	8.9	15.2	13.9	17	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	11.6	18.7
Tetracosanol	262.8	239.8	<LOQ	<LOQ	145.6	180.9	78.2	50.3	73.2	41.2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	5.4	7.1
Hexacosanol	545.2	479.1	11.7	15.6	91.9	162.7	33.7	45.6	103.7	79.5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	18.8	13.6
Octacosanol	727.1	701.8	18.1	20.2	106.9	99.6	32.1	27.3	85.5	73.2	<LOQ	20.7	23.6	18.6	28.1	19	33.4	41.8
Campesterol	584.1	471.6	30.5	35.4	293	309.4	624.8	675.9	485.1	491	1279.9	1423.3	1183.5	1350.9	1151.9	1006.7	166.5	250.3
Stigmasterol	105.8	45.1	10.2	13.7	18.5	15.6	150.1	131.7	1801.6	1705	414.3	383.3	811.8	750.5	87.5	73.5	33.1	28.4
β-Sitosterol	4298.7	3950.6	n.d.	n.d.	2739.6	1891.3	1304.9	1254.6	2101	1893.7	3464.2	3322	2714.5	2451.9	2012.4	1432.9	305.2	289.4
α-Amyrin	98.5	84.2	n.d.	n.d.	<LOQ	<LOQ	n.d.	n.d.	<LOQ	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Oleanolic acid	267.7	234.9	n.d.	n.d.	<LOQ	<LOQ	n.d.	n.d.	205	190.2	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ursolic acid	848.9	803.4	<LOQ	<LOQ	403.2	294.8	<LOQ	<LOQ	261.5	278.4	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

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