Analytical Methods

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Solid phase extraction of α-tocopherol and other physiologically-active components from sunflower oil using rationally-designed polymers

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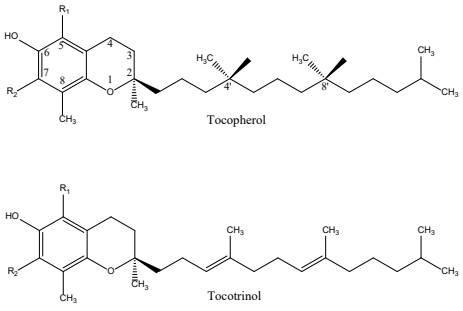
A rationally-designed polymer (RDP) capable of recognition of α-tocopherol and other minor components in sunflower oil has been produced. It is known that sunflower oil is a source of various physiologically-active compounds. Unfortunately, they are present in very minor quantities which make their purification from the complex oil matrix problematic. An extraction method presented here was developed with particular attention to the selectivity, efficiency and precision of the extraction process. The methacrylic acid-based RDP in combination with optimised purification method allowed the extraction of α-tocopherol with 94% recovery. The synthesised polymer was used successfully to extract α-tocopherol together with other essential minor components of sunflower oil without any pre-treatment step. Accordingly to GC/MS, the compounds 'harvested' from sunflower oil using developed polymer included palmitic, oleic and linoleic acids, α-tocopherol, campesterol stigmasterol and β-sitosterol.

Introduction

In 1937, Emerson and his co-worker discovered the group of compounds consisting of α, β, δ and γ-tocopherols that prevent the symptoms of vitamin E deficiency.[1] Nowadays, the formulation which is known as ''Vitamin E'' includes α, β, δ and γ-tocopherols and the corresponding tocoterienols (Fig 1).[2,3] Vitamin E is a mixture of fat-soluble compounds with antioxidant properties which shows promise in preventing and curing Alzheimer's disease, cancer[4,5] and cardiovascular disease.[6,7] It also has a role in protecting cells from oxidative stress through donating electrons to free radicals to neutralise them.[1,7,8] Vitamin E is not synthesised in the human body; therefore, it must be obtained from nutritional sources such as vegetable and seed oils.[9,10] In addition, α-tocopherol is regarded as an important industrial constituent, e.g. it has been used as an additive in the formulation of foods,[11] cosmetics and drugs.[12]

Many attempts have been made to develop efficient and economic procedures for extraction and purification of α-tocopherol and other physiologically-active components from natural oil sources.[12] A majority of oil extraction methods is based on saponification, liquid-liquid extraction and chromatography and require some pre-treatment steps before extraction from oil. All these procedures have also involved several additional steps including solvent extraction, drying and reconstitution steps, therefore, they generate considerable volumes of chemical waste and difficult to control.[10,13] Since α-tocopherol and other bioactive compounds are sensitive to oxygen, light and long exposure to alkaline conditions, these considerations should be taken into account in order to prevent the partial degradation of α-tocopherol and other minor components during their extraction.



	R1	R2
α	CH_3	CH_3
β	H	CH_3
γ	CH_3	H
δ	H	H

Fig. 1 Structure of the eight forms of tocopherols and tocoterienols[7]

Supercritical fluid extraction is one of the techniques that shows promise in terms of comparatively short time scale and low consumption organic solvents, accuracy and economic viability.[1,2] However, it is still generally unavailable for practical applications.[3,5,11]

Solid-phase extraction (SPE) is one of the most effective and popular extraction methods in terms of its low cost and high resistance to environmental and other physical and chemical conditions.[5-7,14,15] It is widely used in industrial and research

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laboratories as an effective clean-up method for bio-recovery of natural compounds from a variety of biomasses.[8] Nevertheless, it is important to highlight that due to complexity and high viscosity of oil matrix, the extraction of any compounds from natural oils is very challenging task. There are very scarce publications that report successful development of the Molecularly Imprinted Polymers as resins for extraction of the oil-soluble pesticides.[16-18] Typically SPE is performed using stationary phase packed in glass or plastic columns. However, the commercial stationary phases are often blamed for their poor stability, which limits a selection of compatible solvents, for inadequate selectivity, limited reusability and restricted binding capacity, especially for polar compounds.[9,10] Therefore, due to the absence of effective commercial resins for extraction of α-tocopherol and other secondary metabolites from oil matrices, there is a demand for an economical, alternative stationary phase that could be cost-effective and could, potentially, be suitable for industrial applications. We would like to demonstrate here the development of bespoke resin for the extraction and purification of a group of minor components including free fatty acids, α-tocopherol and some phytosterols from such complex and dense matrix like sunflower oil. Among the advantages of RDPs which could make them suitable for analytical and industrial applications is their low cost, potential group-specificity towards the compounds sharing some common functionalities, compatibility with mass-manufacturing and high stability.[11-13] We would like to demonstrate here the development of the protocols and materials, which could effectively be applied for the extraction of minor components of sunflower and other vegetable oils.

Materials and methods

Chemicals and reagents

Unrefined sunflower oil was purchased from Activecare through Amazon.com. The vegetable oil-originated α-tocopherol, mixture of phytosterols consisting of 46% β-sitosterol, 24% campesterol and 16% stigmasterol were obtained from Santa Cruz Biotechnology (UK). A sample of free fatty acids containing palmitic, oleic and linoleic acids were purchased from Aldrich (UK). Methanol, ethyl acetate, heptane, acetonitrile, n-hexane, dichloromethane and acetic acid were obtained from Fisher Scientific (UK). All solvents were of HPLC-quality grade and used without any purification. *1,1'*-azobis(cyclohexane carbonitrile), methacrylic acid (MAA) and ethylene glycol dimethacrylate (EGDMA) were purchased from Aldrich (UK). Dimethylformamide (DMF) was obtained from Acros Organics (UK).

Equipment and analysis techniques

The characterisation of the rationally designed polymer was performed using a model solution of α-tocopherol using UV-vis spectrophotometer (Shimadzu, Japan), which was used to quantify the bound α-tocopherol and evaluation the binding capacity of the polymer. 1-mL SPE columns were packed with 100 mg of the RDP and used in combination with a vacuum manifold (Supelco, UK). All SPE experiments were repeated five times. The quantification of α-tocopherol and other minor components was performed using the Gas Chromatography-Mass-spectrometry (GC/MS) set-up (Perkin Elmer, TurboMass, UK) using a 30 m x 250µm, 0.25 mm I.D., ZB-5 capillary column (Phenomenex, UK). Helium gas was used to carry the sample at a flow-rate of 1 mL min^{-1} at 200 °C. After injection of 10 µL of the sample at 200 °C, the temperature of the GC oven was raised by 10 °C min^{-1} to 350 °C and held for 3 min.

Molecular modelling and computational design

The molecular modelling was performed using a workstation from Research Machines running the CentOS 5 GNU/Linux operating system as reported earlier.[19] The workstation was configured with a 3.2 GHz core 2 duo processor, 4 GB memory and a 350 GB fixed drive. This system was used to execute the software package SYBYL 7.3 (Tripos Inc., St. Louis, Missouri, USA). The 2D structure of α-tocopherol and monomers were minimised and Gasteiger-Hückel charges were applied to obtain the 3D molecular structure in Fig 2. A virtual library of 22 functional monomers was screened against the minimised molecular model of α-tocopherol using a LEAPFROG algorithm which was determining the functional monomers that possess natural affinity towards the analyte of interest based on intermolecular interactions such as hydrogen, Van Der Waal or dipole-dipole interactions.[20]

Composition of the RDP

The composition of the polymer was designed based on the results of the molecular modelling using the functional monomer that demonstrated the highest binding energy towards α-tocopherol. Methacrylic acid (MAA) was selected as the functional monomer and ethylene glycol dimethacrylate (EGDMA) as a cross-linker for the preparation of rationally designed polymers (RDPs).

Polymer synthesis and optimisation the monomer cross-linker ratio

Several RDPs were prepared with different monomer: cross-linker ratios (0:100 (P1), 1:99 (P2), 10:90 (P3) and 20:80 (P4)) in order to determine the polymer demonstrating the best performance in the recovery of α-tocopherol. The composition of the polymerisation mixtures is reported in Table 1. All components were dissolved, using ultrasonic bath for 5 min, subsequently the monomeric mixture was deoxygenated by purging with nitrogen for 10 min. The vials containing the monomeric mixtures were tightly closed and allowed to polymerise thermally in a thermostatically-controlled oil bath at 80 °C for 24 h.

Table 1 Compositions of synthesised RDPs with different ratios of monomer: cross-linker

Reagents	Quantity (g)			
	P1	P2	P3	P4
Monomer [a]	0	0.1	1	2
Cross-linker [b]	10	9.9	9	8
Initiator [c]	0.1	0.1	0.1	0.1
DMF	10	10	10	10

[a] MAA, [b] EGDMA, [c] 1, 1'- azobis (cyclohexane carbonitrile)

After polymerisation the monolithic polymer was removed from the vial and ground using a ZM200 Ultracentrifuge Mill (Retsch, UK). The obtained polymer powder was sieved using AS200 Sieve Shaker (Retsch, UK) and a fraction of polymer particles with sizes between 63 to 125 μm was collected. The isolated polymer fraction was washed for 12 h using Soxhlet extraction with methanol. The polymer was dried in the oven at 70 °C. Finally, 1-mL SPE cartridges were packed with 100 mg of polymer and used in extraction experiments. All SPE experiments were repeated 5 times.

Characterisation of RDP:

Measuring the surface area of RDPs

The multi-point Brunauer, Emmett and Teller (BET) method was used to evaluate the surface area of the developed RDPs using Surface Area and Pore Size Analyser (Quantachrome, UK).[21,22] The 45-points isotherm curve was used to evaluate the total pore volume and average pore diameter.

The calculation of the breakthrough volume.

In order to measure the 'breakthrough' volume of the developed polymer a model solution of α-tocopherol in heptane (0.1 mg mL^{-1}) was prepared. SPE was carried out using 100 mg of the polymer attached to a vacuum manifold at a flow rate 0.5 mL min^{-1}. Sequential aliquots of these solutions were passed through the cartridges and the amount of free α-tocopherol left in the filtrates was quantified using a UV-vis spectrophotometer 2100UVPC (Shimadzu, UK) at a wavelength of 296 nm. The breakthrough volume was calculated as the amount of α-tocopherol adsorbed from the fractions that demonstrated ≤ 50% adsorption.[23,24] All experiments were conducted in five repeats.

Calculation the binding capacity

The data of the breakthrough volume was used to calculate the binding capacity (B) of the RDP. One millilitre of a model solution of α-tocopherol in heptane (0.1 mg mL^{-1}) was filtered through 100 mg of polymer. The polymer capacity was calculated using the equation (1):[25]

$$B = (C_i - C_f)\, V\, m^{-1} \quad (1)$$

Where C_i and C_f are the initial and final concentration (fraction correspond to 50% adsorption by the polymer) of free analyte, V is the breakthrough volume and m is the weight of the polymer.

Calculation of the α-tocopherol recovery

This test was done by filtering 1 mL of heptane spiked with 0.1 mg of α-tocopherol (model solution) through 100 mg of the developed RDP. Then, 1 mL of methanol containing 5% acetic acid was used to elute the bound α-tocopherol from the RDP. A comparison between the synthesised polymers and their selection was performed based on the percentages of α-tocopherol recovered from the polymers after the elution process.

Quantification of α-tocopherol

Since the integration values of the area under the peaks in the GC/MS chromatograms were directly proportional to the concentration of the analysed sample, a calibration curve of α-tocopherol was made using the integration of the peaks to calculate the concentration of α-tocopherol in the different fractions of SPE protocol.

Optimisation of SPE protocol for α-tocopherol

The optimisation programme involved choosing different solvents in each step of the SPE process, namely the loading, washing and eluting solvents. The primary selection of solvents was based on literature data that described studies of SPE for α-tocopherol, as shown in Table 2.[3,26]

In order to evaluate the extraction process, the highest percentage of α-tocopherol recovered was used as an indication of the optimal conditions.

Application of the optimised conditions for the extraction of α-tocopherol from sunflower oil

Optimisation of the ratio between the oil and loading solvent

A possibility to use a neat oil in combination with RDP was assessed. Unfortunately, due to the high density of oil it was not possible. Therefore, in order to determine the optimal dilution of sunflower oil in the loading solvent (heptane) 1:9, 1:4, 3:7, 4:6 and 5:5 ratios between oil and heptane were prepared and tested. Sunflower oil samples in heptane were filtered through SPE cartridge packed with 200 mg RDP. The requirements of the washing step were following: the solvent should remove interfering compounds and impurities without losing the compound of interest (α-tocopherol).

Table 2 The solvents tested for each step of SPE

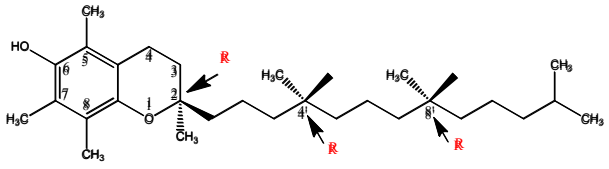
SPE step	Solvents
Conditioning and loading	Hexane, heptane
Wash	Hexane, 50, 60 and 70 % methanol
Elution	Acetonitrile, absolute methanol, methanol with 5% acetic acid

The loaded oil sample, which consisted of 1 mL of 20% (v/v) sunflower oil in heptane, was spiked with internal standards as follows: 300 μg mL^{-1} of palmitic, linoleic acid, α-tocopherol and, mixture of phytosterols (β-sitosterol, campesterol and stigmasterol). The cartridge was washed with 1 mL of 60% methanol, followed by elution using 3 mL of methanol containing 5% acetic acid. The eluted samples were evaporated and then reconstituted in 1 mL of hexane before GC/MS analysis. The percentage of eluted compounds were calculated using the internal standards and corresponding calibration curves. Each experiment was repeated 5 times.

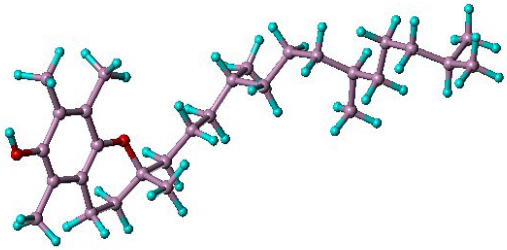
Results and discussion

Molecular modelling

In order to optimise the protocol for the extraction and purification of minor components from oil sample using a developed polymer, a model solution of heptane spiked with a known amount of natural α-tocopherol in heptane was used.



(a)



(b)

Fig. 2 The chiral centres in the 2D molecular structure (a), 3D molecular structure of α-tocopherol minimised using the Sybyl software (b).

It is known that the chemical structure of α-tocopherol has three chiral centres (Fig 2a). Therefore, there are 8 possible stereoisomers of α-tocopherol. However, natural α-tocopherol occurs only in the (2R 4'R 8'R) configuration. A molecular model of α-tocopherol was drawn in configuration of 2R 4'R 8'R using the Sybyl software, then the chemical structure was minimised (Fig 2b).

α-tocopherol was used as a template for the modelling purpose even it hasn't been added to the components of the polymer. The main aim of the 'virtual imprinting' was to select the functional monomers possessing the natural affinity toward the template. Therefore, it was expected that the synthesised polymer might demonstrate some group specificity allowing effectively 'harvesting' compounds with similar functionality and properties from oil matrix.

Screening of the virtual library with the template (LEAPFROG)

The minimised structure of α-tocopherol was used for computational screening of the virtual library of functional monomers using the LEAPFROG algorithm. Each of the monomers was probed for their possible interaction with α-tocopherol. The results of LEAPFROG are reported in Table 3. Using the Sybyl software, it was possible to observe hydrogen bonding between the functional monomers and the template (α-tocopherol), as shown in Fig 3.

Characterisation of the developed polymers

The monomer MAA was chosen for polymer preparation, as it was one of the top candidates from modelling screening and because this monomer has been successfully used in Molecular Imprinted Polymers (MIPs) employed to extract α-tocopherol from non-oil sources.[7,10,27] As it was known from previous studies, MAA has the ability to form hydrogen bonds with α-tocopherol, demonstrating a strong specific binding and also non-specific binding which attributed to long chain moiety and the hydroxyl group in α-tocopherol molecules.[27] The presence of hydrogen-bonding between the functional monomer and α-tocopherol is suggested by Sybyl, as shown in Fig 3. We believe that these two types of interactions (specific electrostatic and non-specific hydrophobic) attribute to high capacity and ability of RDPs to adsorb not only α-tocopherol but also other minor components present in the sunflower oil.

Table 4 Physical characteristics of the synthesised polymers. The measurements were done in 5 replicates

Characteristics	Polymers			
	P1	P2	P3	P4
Breakthrough volume (mL)	9	5	7	4
Binding capacity (mg g^{-1})	8.1±0.7	3.5±0.6	3.3±0.8	2.4±1.0
% recovery	80±2.1	82.6±3.3	94.5±5.2	79.4±3.4
Surface area (cm^2g^{-1})	445.05	437.128	276.15	161.20
Pore volume (cm^3g^{-1})	0.0302	0.0291	0.349	0.240

The EGDMA cross-linker was selected for polymer preparation because it is one of the most commonly used cross-linkers, due to its ability to form rigid polymers with high surface area and, therefore, provide a high binding capacity.[23]

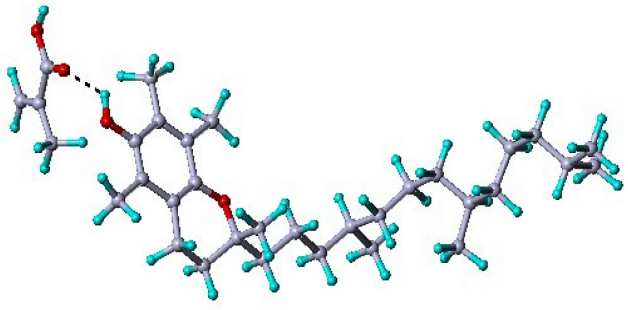


Fig. 3 Molecular complexes between α-tocopherol and MAA functional monomer. The hydrogen bond is shown as a dotted line.

Choosing the optimal monomer: cross-linker ratio

To investigate the effect of the monomer: cross-linker ratio, several polymers were synthesised with different ratio of

monomer to cross-linker (0:100 (P1), 1:99 (P2), 10:90 (P3), 20:80 (P4)). A comparison was made between these polymers in terms of breakthrough volume, binding capacity, recovery, surface area and pore volume as is shown in Table 4. It was observed that all polymers tested were capable of extracting α-tocopherol from the model solution, as revealed by examining the breakthrough volumes and binding capacities. However, although the polymer P1 showed a higher binding capacity than other polymers (8.1 mg g^{-1} of α-tocopherol, breakthrough volume - 9 mL), it was observed that the percentage recovery of α-tocopherol from this polymer was the lowest among other tested polymers. This was attributed to the fact that the polymer was hydrophobic and, thus, difficult to elute. The data obtained in this experiment (Table 4) are recorded as the average of five replicates determinations and standard deviation (SD).

It was found that the polymer prepared using monomer: cross-linker ratio of 1:9 demonstrated the highest recovery percentage of α-tocopherol (94%) (Table 4). The bound α-tocopherol was eluted from the polymers using 3 mL of methanol mixed with 5% of acetic acid (the optimised elution solvent). Using the calibration curve, the concentrations of α-tocopherol after each step of SPE were calculated to compare the percentage of α-tocopherol recovery.

Optimisation of the SPE protocol using the model solution of α-tocopherol

The protocol of SPE using RDPs as adsorbents to extract α-tocopherol was optimised based on previous reported studies[6,10,15,27] that allowed to choose the solvents for each step of the SPE which contributed to the highest recovery percentage of α-tocopherol from the model solution (94%). The optimised conditions, including the conditioning, loading, washing and eluting solvents in all experiments are as follows:

1 mL of the sample in heptane was used for loading, 1 mL of 60% methanol was used for washing and elution was conducted using 3 mL of methanol acidified with 5% acetic acid.

The measurements of α-tocopherol in the different SPE fractions were conducted using GC/MS set up as shown in Fig 4. After loading of the model solution onto the SPE cartridge, it was washed with 60% ethanol, which was consequently evaporated to dryness. Then, the analyte was dissolved in hexane and analysed using GC/MS. Elution of α-tocopherol from the SPE cartridge was achieved with ethanol containing 5% acetic acid. The eluted sample was evaporated and reconstituted in hexane before analysis by GC.

The peak that corresponded to α-tocopherol was analysed using mass spectrometry to check the similarity with a spectrum of α-tocopherol from the spectral library (NIST). As shown in the Fig 5 that there was a considerable similarity between the mass-spectrum of extracted α-tocopherol (upward) and the spectrum of α-tocopherol from the spectral library (downward).

It is known that the list of most commonly used SPE stationary phases for the purification of tocopherol from biological samples or from vegetable oil, and for clean-up before HPLC analysis include following adsorbents: C8[28], C18[15], aminopropyl[29], XAD[15], florisil[29], cyclohexyl[15], Sephadex LH-20[30] or silica gel[19]. However, none of them was reported to be capable of purification of α-tocopherol from oil, either because the analysis protocols require pre-treatment steps[15,29] or adjustment of pH[28] or because the percentage recovery was poor (≤ 20%).[31] Although it was not a goal of this feasibility study, we are confident that a modern technology allows producing the polymer developed here in the industrial quantities and use it to extract α-tocopherol from the oils on the large scale following the optimised extraction protocol that doesn't require any sample pre-treatment or adjustment.

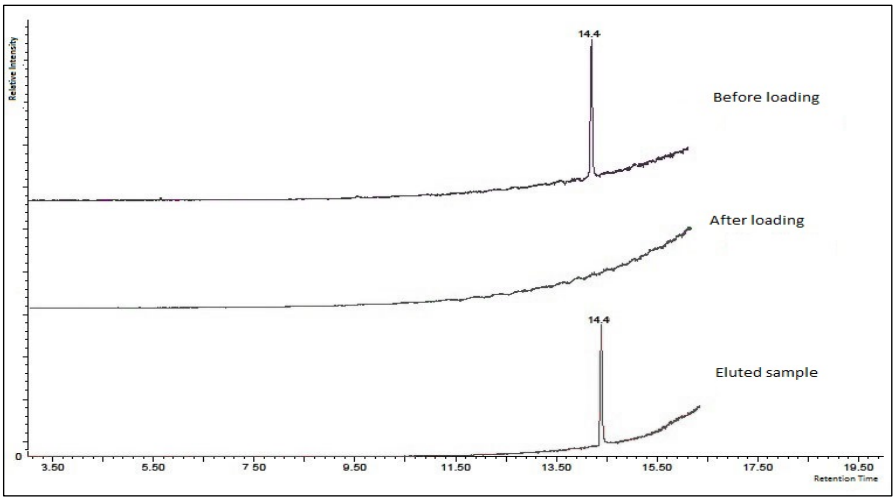


Fig. 4 GC chromatogram of SPE steps of α-tocopherol purification

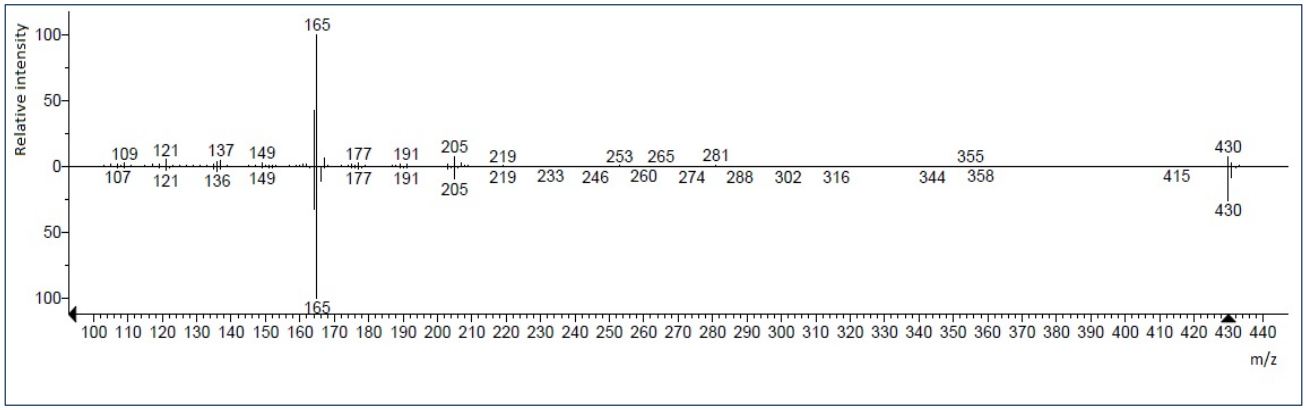


Fig. 5 Mass-spectrum for α-tocopherol purified from model solution (up) and from spectral library (down)

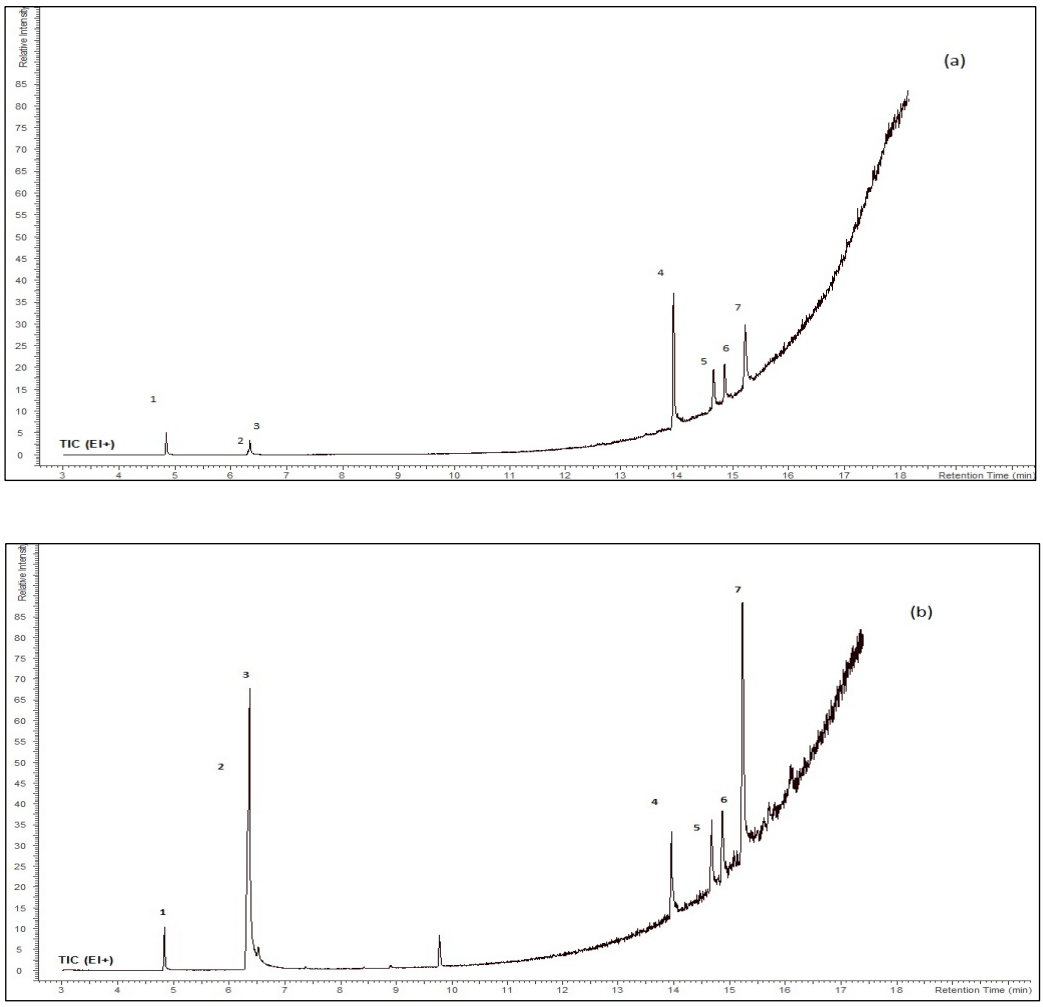


Fig. 6 GC chromatogram of the eluted sample containing a mixture of standards solutions (a) and eluted sample from 20% of sunflower oil (b)

1 palmitic acid, **2** oleic acid, **3** linoleic acid, **4** α-tocopherol, **5** campesterol, **6** stigmasterol and **7** β-sitosterol

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According to the achieved percentage of recovery of α-tocopherol from the model solution, it is evident that the current achievement is comparable with the results from literature.[6-8,10] The benefits of the developed RDP and the optimised SPE protocol includes a minimal dilution of the oil sample, no need in the pre-treatment of the oil and allowed to extract group of essential minor compounds in one step.

Application of the SPE conditions for the extraction of α-tocopherol and other minor compounds from sunflower oil

After the validation of SPE protocol based on high recovery percentage of α-tocopherol from the model solution, the developed RDP and the optimised protocol have been used to harvest the minor components directly from sunflower oil. Thus, the conditions of SPE optimised for model solution of α-tocopherol were applied to a sample of 20% sunflower oil in heptane spiked with 300 μg mL^{-1} of the compounds which were then purified using RDP, eluted and measured using GC/MS. It was observed that it was possible to process it without pre-treatment, which makes it advantageous due to reduced consumption of organic solvents compared to the current industry standards (1:10 dilution ratio between oil and solvent).[8,10] The standard compounds which were used to spike the oil sample included palmitic, oleic and linoleic acids, α-tocopherol and mixture of campesterol, stigmasterol and β-sitosterol. The calibration curves were used to calculate the concentration of the eluted compounds from sunflower oil followed by 5-times dilution in heptane, as shown in Table 5. The eluted compounds are presented as separate peaks in the GC chromatogram (Fig 6b). The eluted compounds were identified using the suggested identifications of the NIST library of mass-spectra. Each peak was analysed by comparison to standard solutions of the compound present in the mass-spectroscopy library (Fig 6a). It was found that the α-tocopherol and phytosterols recovered from the sunflower oil using developed RDP are comparable or even superior to other published reports. For example, the concentration of the α-tocopherol extracted from sunflower oil was more than 485 mg g^{-1} which was reported by Gonzalez (1998).[32] In addition, the content of α-tocopherol in spiked unrefined sunflower oil was found to be close to the published range measured in sunflower seeds by Galea (416 mg kg^{-1})[10] and Ballesteros (473 mg kg^{-1})[33] indicating the efficiency of the developed method. Similarly, the concentrations of phytosterols extracted are in good correlation with the concentrations reported by Lechner who has recovered 325 mg kg^{-1} of campesterol, 198 mg kg^{-1} of stigmasterol and 1868 mg g^{-1} of β-sitosterol.[34] The phytosterols extracted from sunflower oil by Schwartz were reported as campesterol (68 mg kg^{-1}), stigmasterol (280 mg kg^{-1}) and β-sitosterol (2060 mg kg^{-1}).[35]

Table 5 Quantity of eluted compounds from spiked sunflower oil

Eluted compound	Eluted amount (mg kg^{-1})
Palmitic acid	2400 ± 600
Linoleic and Oleic acids	17250 ± 333
α-tocopherol	1138 ± 144
Campesterol	1994 ± 200
Stigmasterol	2705 ± 77
β-sitosterol	5394 ± 38

Conclusions

An effective protocol for the extraction and purification of α-tocopherol beside other minor components from sunflower oil, based on a bespoke RDP, has been developed. The optimised SPE method resulted in increased recovery of the valuable natural product, α-tocopherol from a complex matrix of the sunflower oil. Another important advantage of using the developed polymer over traditional methods of extraction included two-fold reduction in the volume of solvent required. The protocol reported here for the extraction of group of components involved only 5 times dilution of the sunflower oil with heptane. This dilution rate is twice improved by comparison with the 10-fold dilution applied in the industrial protocol, representing a reduction in waste and a saving in the resources and time.

It was also demonstrated that the combination of the optimised SPE protocol and developed RDP allowed a quantitative extraction of minor components from sunflower oil to be performed without any additional pre-treatment. It is important to highlight that the optimised protocols and proposed strategy could be used as blueprints for the development of extraction procedures for different groups of compounds from other natural oil-containing biomasses.

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