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Short communication

Pressurized liquid extraction prior to liquid chromatography with electrochemical detection for the analysis of vitamin E isomers in seeds and nuts

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Abstract

Pressurized liquid extraction (PLE) was used to isolate tocopherols from seeds and nuts. Very clean extracts were obtained, which were injected directly into the chromatographic system. This enables rapid and simple control in food analysis. The PLE extraction temperature was set at 50 °C, with two cycles of extraction, a static time of 5 min, and acetonitrile as the extraction solvent. LC separation was accomplished on a Synergi Hydro-RP column with methanol–water (99.9:0.1, v/v) containing 2.5 mM acetic acid/sodium acetate buffer, as eluent. Coulometric detection, with a porous graphite electrode at +700 mV, was used. The method was successfully applied to the determination of α-, (β + γ)- and δ-tocopherols in almonds, sunflower seeds, hazelnuts and walnuts. The recoveries were in the 82–110% range. The results were validated with those obtained using sample treatment including alkaline hydrolysis.

Keywords: Food analysis; Seeds and nuts; Tocopherols; Pressurized liquid extraction; Coulometric detection

1. Introduction

Pressurized liquid extraction (PLE) is an extraction technique that uses an organic solvent or mixture of solvents at high pressure and a temperature above their boiling points to extract substances from a solid matrix [1,2]. PLE has been developed as an alternative to Soxhlet extraction, reducing the volume of extraction solvent and shortening the time required for extraction. In PLE, a solid sample is packed into the extraction cell and is extracted with a solvent at elevated temperature. This implies that the pressure inside the extraction cell must be kept high enough for the solvent to remain in the liquid state.

PLE has been used in the analysis of contaminants in soils, such as organochlorine and organophosphorus pesticides, polycyclic aromatic hydrocarbons and polychlorinated biphenyls [3]. The technique also offers possibilities for the extraction of micronutrients, such as liposoluble vitamins from food samples, although it has rarely been used in this field. It is important to note that substances not naturally present in food, such as pollutants, are more easily extracted than substances that are naturally present. As a lipophilic substance, vitamin E is intimately associated with the lipid components of the food matrix.

Commonly used methods of sample preparation in the case of liposoluble vitamins include sample saponification and solvent extraction [4–7]. In food analysis, sample preparation can also be achieved by direct liquid–liquid extraction without saponification, especially with an organic solvent such as hexane [8–12], anhydrous ethanol or methanol [13], or mixtures such as hexane–2-propanol [14–16], acetonitrile–methanol–2-propanol [17]. In most cases, after extraction a purification step is necessary, and the extraction process is not always carried out with sufficient efficiency. A sample preparation method in continuous mode involving direct extraction of the analytes through a sil-

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icone membrane coupled on-line with the chromatographic system has also been used [18].

The chief objective of the present work was to develop a new method for the extraction of vitamin E isomers from seeds and nuts. The method included PLE and direct injection into a liquid chromatographic system with no cleaning step. Detection was carried out with coulometric detection (electrochemical detection, ED) [18]. The method was used for the analysis of vitamin E in seeds and nuts, which are a main source of this vitamin.

2. Experimental

2.1. Apparatus

2.1.1. LC system

Isocratic LC was carried out with a Spectra-Physics (San José, CA, USA) SP8800 ternary pump, equipped with a Rheodyne (Berkeley, CA, USA) valve with an injection loop of 20 μL and a Phenomenex (Torrance, CA, USA) Synergi 4 μ Hydro-RP 80A column (250 mm × 4.6 mm i.d.). An ESA (Chelmsford, MA, USA) 5200 A coulochem detector with an ESA 5020 guard cell connected to the system to treat the mobile phase before injection (E = +1000 mV) and an ESA 5010 analytical cell containing dual porous graphite working electrodes, was used (E_1 = −500 mV, E_2 = +700 mV). Data were processed on a Shimadzu (Duisburg, Germany) CBM 10-A communication module and a computer using Class-LC10 Software (Shimadzu).

2.1.2. PLE

PLE (Dionex trade name: ASE for accelerated solvent extraction) was performed on a Dionex (Idstein, Germany) ASE 100 automated extraction system. Extraction cells of 34 mL, with cellulose filters and Dionex vials of 250 mL for extract collection were used.

A Büchi (Flawil, Switzerland) RE 121 rotavapor with a Büchi 461 water-bath were used. Water was purified on an ElgaStat water purification system (Elga, High Wycombe, UK).

2.2. Reagents

The following compounds and chemicals were used: α-tocopherol (>98% Sigma, Alcobendas, Madrid, Spain), γ-tocopherol (Sigma), δ-tocopherol (90% Sigma), β-tocopherol (Merck, Darmstadt, Germany), special HPLC-quality acetonitrile (Merck), special HPLC-quality methanol (Merck), special HPLC-quality *n*-hexane (Merck), R.A.-grade glacial acetic acid (Scharlau, Barcelona, Spain), R.A.-grade sodium acetate (Pancreac, Barcelona, Spain), R.A.-grade potassium hydroxide (Scharlau), R.A.-grade ascorbic acid (Panreac), R.A.-grade ethanol (Scharlau). Hydromatrix Celite Varian (Scharlau), Florisil adsorbent for chromatography (Fluka).

All parameters that affect the extraction process were studied with raw almond samples and the methodology developed was applied to almonds, sunflower seed, hazelnuts and walnuts. All samples were from commercial sources.

The mobile phase was a solution of 2.5 mM acetic acid/sodium acetate in MeOH/H_2O (99.9:0.1, v/v). The flow rate was set at 1.5 mL/min. The solution was filtered through Millipore (Madrid, Spain) nylon membranes with a pore size of 0.22 μm and a diameter of 45 mm, and was degassed with helium.

2.3. Sample treatment

2.3.1. Sample treatment using PLE

The ASE system consisted of a stainless-steel extraction cell with electronically controlled heaters and pumps to maintain the extraction parameters following an established programme. The general procedure was as follows: ground samples (approx. 1 g) were mixed homogeneously with a drying agent (Hydromatrix Celite) in the extraction cell. After placing the cell in the PLE system, the optimised programme was applied. This included a temperature of 50 °C, static time of 5 min (two cycles) at a pressure of 1600 psi (1 psi = 6894.76 Pa), using acetonitrile as solvent. The extracts were brought to 50.0 mL, and an aliquot of this solution (4.0 mL) was filtered through 0.22 μm nylon membranes and diluted to 10.0 mL. This solution was injected (20 μL) into the LC system. Analyte elution was complete in 12 min.

2.3.2. Sample treatment with saponification of samples

The ground samples (approx. 2 g) were saponified, protected from light at room temperature, over 2 h under stirring with an alcoholic solution of potassium hydroxide plus ascorbic acid to avoid the oxidation of liposoluble vitamins. The composition of the hydrolysis reagent was as follows: 50 mL of ethanol, 5 mL of aqueous 10% ascorbic acid, 10 mL of aqueous 80% KOH, and 25 mL of water. The analytes were then extracted with hexane (2 × 25 mL) and the extracts washed with water (2 × 10 mL). The organic phase was removed by evaporation in a rotavapor under vacuum at 50 °C, and the residue dissolved in methanol (50 mL) and filtered through Millipore nylon membranes with a pore size of 0.45 μm to clean the extracts before injection.

3. Results and discussion

3.1. Analytical characteristics of LC–ED

The vitamin E isomers were quantified using the external standard method. Linear calibration plots were obtained for all isomers (r^2 = 0.999). The limits of detection (S/N = 3) were 10, 9.5 and 12 μg/L for δ-, γ- and α-tocopherol, respectively. The R.S.D.s obtained in a study of inter-day precision (n = 10) for a standard sample containing δ-, γ- and α-tocopherol at about 100-fold higher concentrations were

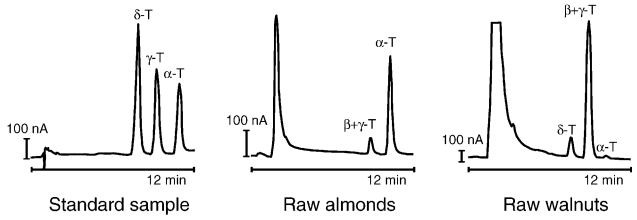

Fig. 1. LC–ED of methanolic solutions of standards and two samples. PLE and LC conditions as in proposed method (see text).

3.4, 2.9 and 3.4%, respectively. Fig. 1 shows a chromatogram obtained for a standard solution.

3.2. Optimisation of PLE parameters

3.2.1. Solvent extraction

Acetonitrile was chosen as the solvent for extraction because the analytical signals obtained were higher than when using methanol. Traces of lipids were extracted from the matrix, and hence a dilution step was included in the procedure to avoid column poisoning. This dilution is possible owing to the high sensitivity of ED.

3.2.2. Sample amount

The extraction cell in the PLE system contains the sample mixed with a drying agent to avoid a dead volume in the cell and to allow the best contact of sample and solvent. To achieve the best analyte extraction conditions, amounts of sample ranging from 0.5 to 4 g were weighed and mixed with the drying agent (Hydromatrix Celite) until the extraction cell was full. For sample amounts above 3 g, the signal tended towards a constant value. Because the relationship between sample amount and analytical signal must be linear, it was necessary to work in the ascending part of the curve, and amounts of 1 g were used. Some tests using mixtures of Hydromatrix Celite and Florisil adsorbent were performed in order to avoid traces of lipids in the extracts, but the extracts obtained were similar both with and without Florisil.

3.2.3. Extraction temperature

Temperature is an important parameter in PLE. High temperatures increase the efficiency of the extraction, but vitamin E isomers are thermally unstable. Accordingly, only values of 40–70 °C were tested, and no great variation in the analytical signal was observed in that range, although lower signals were seen at 70 °C. A temperature of 50 °C was therefore chosen.

3.2.4. Static time and number of extraction cycles

To study the static time, extractions were carried out from 1 to 10 min. Long times slightly increased the analytical signal, but they distinctly increased the cycle time. A static time of 5 min was therefore chosen as an acceptable compromise between signal intensity and time of analysis.

The influence of the number of extraction cycles was studied by applying the procedure to almonds, using 1, 2 or 3 extraction cycles. The values obtained using 2 or 3 cycles were highest but mutual differences were small. To save time, two cycles were used in all further work.

3.3. Determination of tocopherols in seeds and nuts

Seven samples of various seeds and nuts were analysed. These included raw and roasted almonds, raw and fried sunflower seeds, raw and roasted hazelnuts, and raw walnuts. The samples had different tocopherol contents. The experimental results are shown in Table 1. Fig. 1 shows chromatograms obtained for samples of raw almonds and raw walnuts.

It should be stressed that the peak appearing at 8 min when seeds and nuts were being analysed could correspond to β- and γ-tocopherol. It is known that the positional β- and γ-tocopherol isomers cannot be completely resolved by reversed-phase LC. Normally, β-tocopherol levels in these samples are lower than those of γ-tocopherol. Only in a few cases is the β- isomer concentration important compared with that of the γ-isomer.

To determine intra-day precision, raw almond samples were extracted and analysed six times on the same day under the same conditions; the R.S.D.s were 3.0 and 2.3% for γ- and α-tocopherol, respectively. The inter-day precision was also obtained; the R.S.D.s in this case were 12 and 7.3% for γ- and α-tocopherol, respectively.

To test the recovery, analyses were carried out on spiked samples of each seed or nut. In all cases the amount of each vitamin E isomer added was close to that in the original sample. Recoveries ranged from 82 to 110% (Table 1).

Table 1
Analysis of vitamin E isomers in seeds and nuts using PLE and LC–ED ($n = 3$)

Sample	Vitamin E isomer (mg/100 g)			Recovery (%)[a]		
	δ-T	(β + γ)-T	α-T	δ-T	γ-T	α-T
Raw almonds	–	1.45 ± 0.05	18.2 ± 0.5	–	99	104
Roasted almonds	–	1.66 ± 0.02	22.3 ± 0.3	–	90	98
Raw sunflower seeds	–	1.29 ± 0.04	26.9 ± 0.6	–	100	105
Fried sunflower seeds	–	2.07 ± 0.07	16.4 ± 0.7	–	90	82
Raw hazelnuts	–	0.76 ± 0.04	9.0 ± 0.4	–	82	99
Roasted hazelnuts	0.90 ± 0.04	5.35 ± 0.04	22.1 ± 0.7	92	98	92
Walnuts	3.05 ± 0.07	19.5 ± 0.4	2.6 ± 0.1	110	102	105

[a] Spiked concentration of each analyte close to that in original sample.

3.4. Comparison between methodologies

Using the procedure described in Section 2.3.2, almond samples were analysed. This is the classical procedure for the analysis of liposoluble vitamins in milk, butter, oil, etc. [19], and involves alkaline hydrolysis followed by extraction with an organic solvent. The classical and the present method were compared using Student's *t*-test for unpaired values. The levels of significance obtained were 0.1062 and 0.5545 for γ- and α-tocopherol, respectively. In both cases, the value was higher than 0.05 (chosen as the minimum level of significance). The null hypothesis was accepted, and no differences among the results were observed.

4. Conclusions

The present study shows that PLE can be used to isolate naturally occurring tocopherols from seeds and nuts with the advantages inherent to this technique such as a small amount of solvent and speed. The proposed method requires no additional cleaning after the PLE step, final analysis by LC–ED gives good results. The time of analysis is 35–40 min after weighing the samples. These characteristics allow the method to be applied in routine analysis.

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References

[1] B.E. Ritcher, J.L. Ezzell, W.D. Felix, K.A. Roberts, D.W. Later, Am. Lab. 27 (1995) 24.
[2] B.E. Ritcher, B.A. Jones, J.L. Ezzell, N.L. Porter, N. Avdalovic, C. Pohl, Anal. Chem. 68 (1996) 1033.
[3] H. Giergielewicz-Mozajska, L. Dabrowski, J. Namiesnik, Crit. Rev. Anal. Chem. 31 (2001) 149.
[4] F.J. Rupérez, D. Martín, E. Herrera, C. Barbás, J. Chromatogr. A 935 (2001) 45.
[5] J. Parcerisa, D.G. Richardson, M. Rafecas, R. Codony, J. Boatella, J. Chromatogr. A 805 (1998) 259.
[6] F. Lavedrine, A. Ravel, A. Poupard, J. Alary, Food Chem. 58 (1997) 135.
[7] A. Demo, C. Petrakis, P. Kefalas, D. Boskou, Food Res. Int. 31 (1998) 351.
[8] J. Van der Greef, A.J. Speek, A.C. Tas, J. Schriver, M. Hoehn, U. Rapp, LCGC 4 (1986) 63.
[9] F. Yao, G. Dull, R. Eitenmiller, J. Food Sci. 57 (1992) 1194.
[10] K.S. Epler, R.G. Ziegler, N.E. Craft, J. Chromatogr. B 130 (1993) 37.
[11] D.B. Gomis, M.P. Fernández, M.D.G. Alvarez, J. Chromatogr. A 891 (2000) 109.
[12] A. Schieber, M. Marx, R. Carle, Food Chem. 76 (2002) 357.
[13] B. Deng, P. Chen, X. Cai, Gaodeng-Xuexiao Huaxue Xuebao 9 (1998) 333.
[14] G.J. Guzman, P.A. Murphy, J. Agric. Food Chem. 34 (1986) 791.
[15] A. Abdollahi, N.S. Rosenholtz, J.L. Garwin, J. Food Sci. 58 (1993) 663.
[16] G.W. Chase Jr., C.C. Akoh, R. Eitenmiller, J. Am. Oil Chem. Soc. 71 (1994) 877.
[17] E.J. Rogers, S.M. Rice, R.J. Nicolosi, D.R. Carpenter, C.A. Mc Clelland, L.J. Romanecyk, J. Am. Oil Chem. Soc. 70 (1993) 301.
[18] M.M. Delgado-Zamarreño, M. Bustamante-Rangel, A. Sánchez-Pérez, J. Hernández-Méndez, J. Chromatogr. A 935 (2001) 77.
[19] Official Methods of Analysis of the AOAC, 15th ed., Association of Official Analytical Chemists, Arlington, VA, 1990.