International Journal of Engineering Research & Technology (IJERT)
ISSN: 2278-0181
Vol. 4 Issue 02, February-2015

Analysis of Melatonin by High Performance Liquid Chromatography after Solid-Phase Extraction (SPE/HPLC-FD)

Najoua El Moussaoui [1,2], Abdenbi Bendriss[2]
[1]Department of Analytical Chemistry, Faculty of Sciences, University of Cadiz, Spain.
[2]Department of Biology,Faculty of Sciences, Abdelmalek Essaadi University,Tétouan 2121, Morocco

***Abstract*-The solid phase extraction (SPE) procedure on octadecylcilica (C18) and styrene-divinylbenzene cartridges was developed for the determination of melatonin. It was studied the influence of several extraction variables; including three different solvents (water, methanol and ethanol); the solid phase employed and the eluting volume (1, 2 and 3mL). The linear range of the melatonin standard curve was from 0.05-1 mg/L (R^2> 0.997). The HPLC-FD method was validated as reliable for the quantitative analysis of melatonin standard that met AOAC requirements: LOD =0.075mg.L^{-1} and LOQ=0.25mg.L^{-1}.Repeatability and reproducibility of the developed method was established. Also robustness was checked regarding the main extraction variables. All the recoveries of melatonin from several types of solid phase and solvent were obtained under the optimized conditions for final analysis by HPLC with fluorescence detector.**

***Keywords:*Solid phase extraction; melatonin; wine; solid phase.**

I. INTRODUCTION

Melatonin (N-acetyl-5-metoxytryptamine) is a neurohormone produced by the pineal gland and, it has also been found mainly in foods of plant origin. The literature on the biological properties is very wide, so it has increased substantially the interest of their study in different types of plants and fruits.

It is widely recognized that dietary intake of fruit and a vegetable is important in order to maintain good health. Programmes such as “Five a day” have been introduced to increase consumer consumption of these natural products and their bioactive ingredients [1]. This molecule is a biogenic indoleamine, wich performs an important role in the regulation of circadian rhythm [2] and the alleviation of sleep disorders, such as insomnia due to jet-lag and shift work [3; 4] . It has also been reported to have potent anti-oxidative properties and anti-inflammatory effects [5; 6]. In addition, melatonin may also act as a protective agent in ocular diseases [7] (Siu et al. 2006) and also acts as an anticancer agent [8].Some literature reviewed suggests that melatonin might also have the function of countering high levels of free radicals that may be generated by metabolic activities. Likewise direct exposure to sunlight or UV light that promotes photo-oxidation could also have an influence on the biosynthesis of melatonin in plants, increasing its production levels [4; 9]. The presence of melatonin in food can be determined and confirmed by different extraction protocols, together with efficient analytical methods, because traditional analytical methods have a low specificity. Melatonin can be detected by several methods, such as immunological techniques, Radioimmunoassay (RIA) and Enzyme-Immunoassay (EIA) [10].

Aiming to increase the specificity of the methods has been also used liquid chromatography with different detectors: electrochemical [11], fluorimetric [12] and MS / MS [13]. Another possible alternative is capillary electrophoresis in different variants, which has the advantage of low values of limits of quantification [14; 15] warn that the matrix effect of plant samples due to the presence of reducing agents may interfere in the analysis.

The chromatographic techniques are more economical and time efficient when derivatization of the sample is not required prior to analysis. Most of the HPLC methods reviewed have used reverse phase columns (e.g. RP18 or RP8) for melatonin separation and fluorescence detectors (FD) were found to be sensitive and versatile to quantify melatonin in food samples and also gave low limits of detection and quantification. Several methods for the extraction of melatonin from vegetable and food samples have been reported, and these no include ultrasound-assisted extraction [11], liquid-liquid extraction [13].

Solid phase extraction is a good choice for the extraction/concentration of aromatic compounds since several solid phases are available which allows for increasing selectivity in the extraction process. Moreover, great enrichment of the aromatic compounds in the extract can be achieved by using a small amount of organic solvent during the elution step from the solid phase. SPE has previously been used for the extraction of aromatic compounds from different samples [16; 17].To the isolation and pretreatment steps of pesticide residues in food and environment matrices [18]. It is has been used also, for volatile compounds in wine, for determining pesticides by SPE-GC-mass spectrometry (MS) using a C-18 solid phase; and for the simultaneous determination of 2, 4, 6 trichloroanisole (TCA) and 2, 4, 6-tribromoanisole (TBA) in wines [19]. Another multiresidue method based on solid-phase extraction was developed for the simultaneous determination of 50 pesticides in commercial juices [20].

The type of matrix has an important influence on the particular sample preparation and in the case of fruit juices, a method for the simultaneous determination of folpet, chlorothaloni, quinomethionattetradifon and trifluralin has been developed by using C-18 SPE cartridges for sample purification and pre-concentration [21; 22], and matrix solid-phase dispersion (MSPD) [23] have been used with good results. Selection of the most adequate solid phase is the most time-consuming part of the method development.
Although SPE (to our knowledge),never has been used for a melatonin standard solution using thestyrene-divinylbenzeneSPE. An extensive study of the ability of different solid phases to retain aroma compounds from wines has been made, but styrene–divinylbenzenephase (P1) was not evaluated, it has various attractive features.
The objective of the present work was todevelop solid-phase extraction method for determination of melatonin standard recovery; different solid phases (based onC-18andpolystyrene-divinylbenzene) had to be studied and also the solvent providebetter results for theselectiveretention andelution of the melatonin. All those variables optimized to guarantee complete recovery of melatonin standard.

II.MATERIALS AND METHODS

A. Chemicals and reagents

Melatonin standard was purchased from Sigma-Aldrich™ (St Louis, MO.USA). Methanol and Ethanol (HPLC grade) from Scharlau Chime, Barcelona; glacial acetic acid for analysis was purchased from Merck (Darmstadt, Germany). Solutions were prepared by dilution in Milli-Q water produced using a Millipore water purification system coupled to a Milli-Q module (Millipore Bedford, MA).

In other work, Gergely. A, has been evaluated that the pigments of red wines can be preconcentrated on SPE cartridges containing octadecylsilica sorbent, and the preconcentration step makes possible the separation and detection of pigment fractions present in low concentrations in red wines by HPLC [24]. SPE combined with HPLC/MS; it has been used for a determination of melatonin in medicinal plants using the C-18 SPE cartridge (5mL, C18, 200mg) [25].

B. Standard solution and sample preparation

A 0.5mg.L^{-1} of melatonin standard was prepared in 100% of Milli-Q water; it was prepared also in ethanol and methanol 12 % (v: v) in water; to study the solvent providebetter recoveries (%) results of the melatonin standard solution.

C. Solid phase cartridges

Seven solid phase cartridges were evaluated for the extraction of melatonin standard and melatonin from wines. Table 1 shows their main characteristics and codes used for them.

Table 1.Characteristics of solid phase cartridges.

Solid phase	C-18				Styrene-divinylbenzene		
Identification	C-18 1	C-18 2	C-18 3	C-18 4	P 1	P 2	P 3
Supplier	Strata C18 E	Lichrolut RP-18	Discovery DSC-18	Bond Elut C18	Strata-X	Strata SDB-L	Lichrolut EN
Manufacturer	Phenomenex	Merck	Supelco	Varian	Phenomenex	Phenomenex	Merck
Amount of solid phase (mg)	500	500	500	500	200	200	200

D. Determination and extraction of melatonin

- *Solid-Phase Extraction (SPE)*

RapidTrace (registered mark). Zymark, Hopkinton, Massachussets, EEUU. Is a modular, highly scalable usingcartridges solid phase extractionat industry standardofV =3mL.The system cansupport 3mL (ten positions); each modulecan be loaded withten cartridges.

The modular designlets themaddproduction capacityas needed.Modules withinagroupcan operatewith different methods;the product isdesigned to maximizepreparation stepsand the cleaningofthe sample beforethe sample analysisbyliquid chromatography(LC). Rapidtracespecializesin the automation ofsolid phaseextractionoflow volume.

TheSPEremains theprocedureofsample preparation offastergrowth. It ismoreusualinthe samples concentration and treatmentprior toanalysisbyHLC, HPLC / MS, GC O GCMS.It has been proven that SPE offered several significant advantages over liquid- liquid extraction (LLE), such as less consumption of organic solvent, shorter analysis time, no phase emulsion, higher method recovery, and more efficient removal of interfering compounds.

- Chromatographic conditions: HPLC-FD

Chromatographic analyses were carried out on an Dionex System with pump system P680, an ASI 100 autosampler, a column oven TCC 100, a UV-visible detector array of photodiodes aligned PDA 100 and an RF 2000 fluorescence detector. The column used in this study was Lichrospher®100; RP-18(5µm), 250*3mm, at a room temperature of 25ºC. Chromeleon 6.60 chromatographic software was used for HPLC control and peak integration.

A gradient elution was used with two mobile phases: phase A (2% acetic acid and 8% methanol in water) and phase B (2% acetic acid and 90% methanol in water).Isocratic elution was used applying 50/50 (A: B) at a flow rate of 0.5 mL/min and injection volume of 10µL.

The spectra wererecordedusingadiode arrayalignedand1.2 nmresolutionscanningfrom 240 to390nmandawavelengthλex=280nmandλem =310nm.The HPLC mobile phases were first degassed in an ultrasonic bath and have filtered through a 0, 45 µm membrane before analysis with HPLC-FD.

E. Extraction procedures

- Rapid Trace conditioning

Firstly, all the solid phases were activated by passing 5mLofmethanol and5mLof water through them. The sample was passed through the activated sorbent at around 0.5mL/min and the sorbent was then washed with 5mLof water, melatonin was recovered by elution with 2mL of methanol and, finally, rinsed with6mLofmethanol, the extract was dissolved at 5mLin Milli-Q water,then was filtered with filters 0. 45µm nylon and passed directly by HPLC-FD.

- Solvent and solid phase selection
  - ✓ Solvent

In this paper we have focused our work to study two different variables: solid phase cartridge and solvent; in order to choose the combination to provide better results for the selective retention and elution of the melatonin standard and melatonin in wine (spiked sample). Three different solventswere used: Water (H_2O), ethanol (Et-OH) and methanol (Me-OH).

To develop themethod has been used0.5mg.$L^{-1}$of melatonin standard. This standardwas dissolved inthreedifferentvolumetric flasks: **1**-the first with 100%of water(100% H_2O), **2**- the second with a mixture of ethanol-water (12:88% /ethanol in water) and the last one 3- with a mixture of the same volume methanol-water (12:88% methanol in water). All results of melatonin recoveries are shown Graph 1 (a; b and c).

  - ✓ Solid phase

We have studied2kinds ofsolid phasesin the form of seven cartridgesthancommercialsolid-phase extraction, four of them based onasolid phaseoctadecylcilicaC-18(C-18 1; C-18 2; C-18 3 and C-18 4),other threeconsisting ofpolymersof styrene-divinylbenzene (P1; P2 and P3).The characteristics ofeach solid phaseare presentedinTable1previously mentioned.
Graph 2shown the melatonin recoveries (%) of six cartridges (C18.1; C18 2; C18 3; C18 4; P1 and P2) after removing P3 as the worst cartridge; proceed to the next step using the above process with the same object of removing more cartridges that produce a low melatoninrecovery.

- Method development

For the method of development(selection ofadsorbentand solvent); all cartridges were conditionedbefore each use, with5mLofmethanol and 5mLofwater. Thenthe solutionis preparedto be passedby each ofthese.To do this, are added5mLof melatonin standard solution;this solution was prepared in a waythat hadaconcentrationadequate to provideameasurable signalin thechromatogram.

Once, ithas passed thedissolutionof melatonin andensure that thereis stillbeing retainedinthe solid phase, 5mLof waterare addedand then6mLofmethanolto discard theremains ofmelatoninthat couldpresent the wineto be analyzed.Thentheanalyteis elutedwith5mLofwater. Finally, the extracts obtained were diluted at 5mLof Milli-Q water, then were passed through filters (0. 45µm) and injectedwith an auto- samplerin theliquid chromatographyequipment (LC). All assays wereperformed in duplicate.

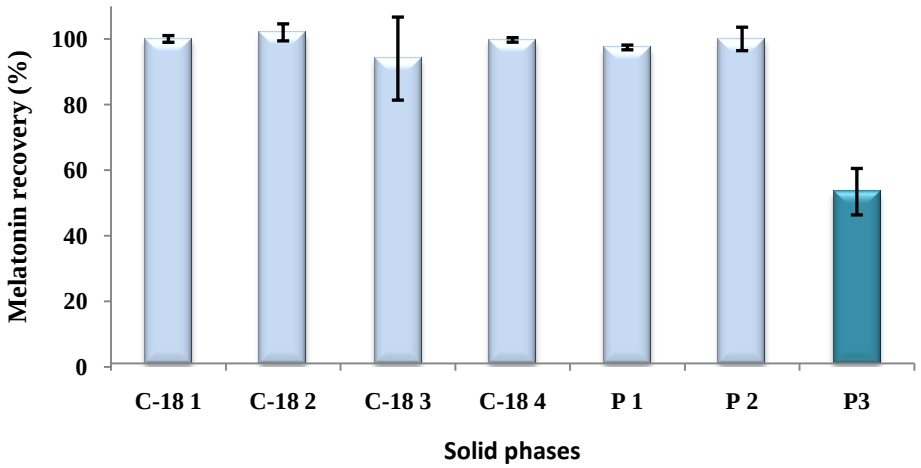
III. RESULTS AND DISCUSSION

- Solvent and solid phase selection

Two varieties of solid phases were studied, in seven different commercial solid phase extraction cartridges; four of them based on octadecylcilica (C18) and the others three based on styrene-divinylbenzene,in order to evaluate the best solid phase and the best solvent, to ensure the best conditions extraction in the followings works in real samples.Characteristics and codes used for them as described in Table 1; and conditioned as described in Section 2 (2.3).

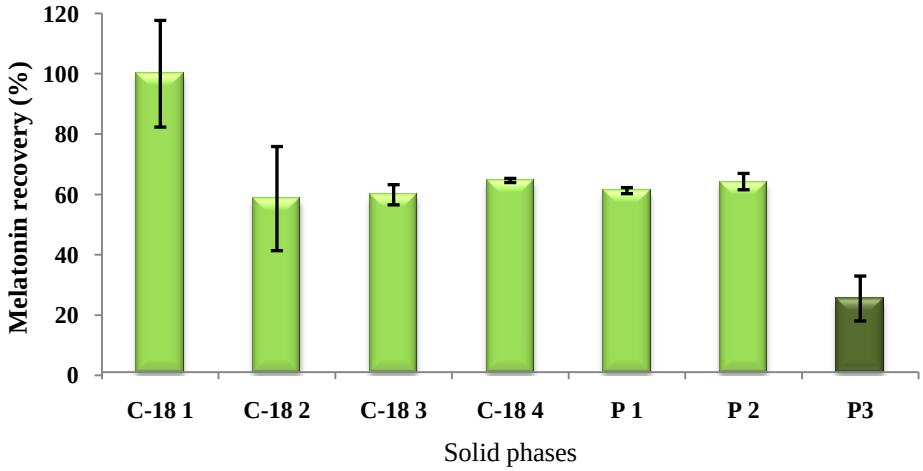
All results shows the average recovery (%) of melatonin using the three various solvents (water, ethanol and methanol) and the seven solid phases used in the study are presented in Graph 1 (a, b and c).
Graph 1 (a)shows the recoveriesobtainedfrom the seven solid phases usedaccording to 100% of water solvent.The results found that the P3was the worst solid phase with

poorrecovery53%, while other solid phases found acceptable recoveries ≥ 94%.



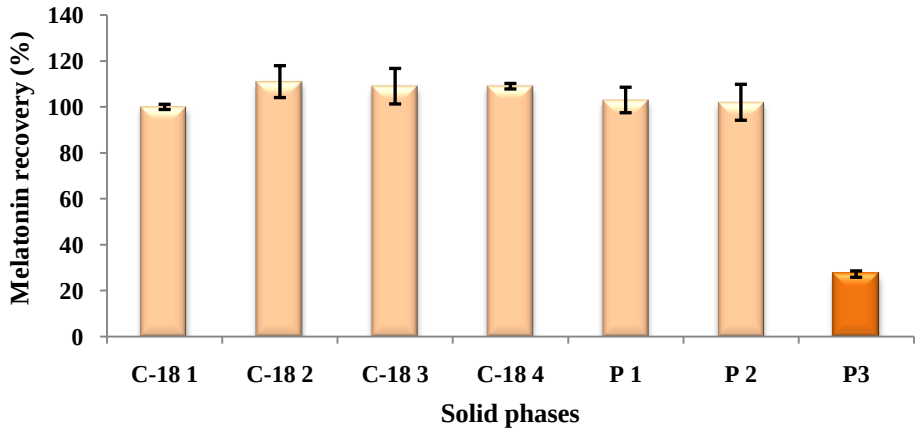
Graph 1 (a).Melatonin standard recoveries (%) by SPE using seven solid phase cartridges and 100% of water solvent.

Thereafter, we have evaluated the effect of other solvent; as 100% of water is changed by the mixture ethanol-water 12:88 (v: v) while the solid phases are the same. The results obtained are shown in graph 1 (b).We note that melatonin recovers 100% in the first cartridge C-18, and more and less 59% in the next cartridges except the last cartridge P3 recovery not exceeding 26%.



Graph 1 (b).Melatonin standard recoveries (%) by SPE using seven solid phase cartridges and 12% of ethanol solvent.

It remains the third solvent, in order to choose the best one among the three studied; it has been the mixture of methanol-water 12:88 (v: v). Graph 1 (c) shows the results obtained. As it can be seen, there are not many differences betweenresults obtained using 100% of water and methanol/water (12:88); the first six solid phase cartridges and all have higher recoveries ≥ 100%, except the P3 solid phase that has the lowest recovery 27%.

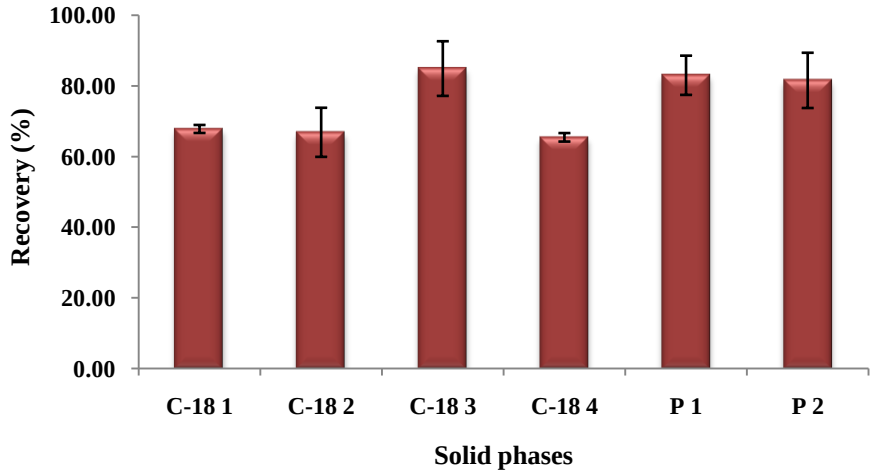


Graph 1 (c).Melatonin standard recoveries (%) by SPE using seven solid phase cartridges and 12% of methanol solvent.

Therefore, considering the results presented in the Graph.1 (a, b and c);methanolwas selected the best solvent producing the highest recoveries ≥ 100%for all the six solid phases. After removing P3 as inadequate solid phase for extraction and choose the best solvent such asmethanol, we continued the same extraction method with methanol, and the cartridge that produced the lowest recovery is automatically removed.

Graph 2 shows the average recoveries (%) obtained using the six solid phases (except P3 previously removed). We observe that among the six solid phases,only three produced good recoveries: C-18 3; P1 and P2, recoveries >81% were obtained for these cartridges; while the recoveries of three others did not exceed 68%.

Based on the recoveries obtained for C-18 3, P1 and P2 using methanol as solvent was chosen the second one (P1) as the most adequate solid phase cartridge, although there were no many differences from P2 and C-18 3.



Graph 2.Melatonin standard recoveries (%) by SPE usingmethanol as solvent.

In the SPE, two amounts of solid phase were checked: 200 and 500 mg.According to the recoveries obtained are shown in graphs 1 and 2.As can be see that the higher recoveries were found for the 200 mg cartridges.

IV. CONCLUSION

All the results obtained in this study found that the combination of Strata-X cartridge and methanol with the elution volume 1mL is the best to extract melatonin and was selected for the method optimization. Therefore, the developed SPE method was satisfactorily applied to real samples.

ACKNOWELDGEMENTS

Authors thank all Profs and equip researchers from chemistry department of the University of Cadiz, for allowing us to carry out HPLC determination with fluometric detection and solid phase extraction using their equipment.

REFERENCES

1. GeeromsNele, WimVerbeke, Patrick Van Kenhove (2008). Health advertising to promote fruit and vegetable intake: Application of health-related motive segmentation .Food Quality and Preference, 19 (5), 481-497.
2. Srinivasan, V., Singh, J., Pandi-Perumal, S.R., Brown, G.M., Spence, D.W., &Gardinali, D.P. (2010). Jet lag, circadian rhythm sleep disturbances, and depression: The role of melatonin and its analogs. Advances in Therapy, 27(11), 796-813.
3. Buscemi, N., Vandermeer, B., Hooton, N., Pandya, R., Tjosvold, L., Hartling, L., et al. (2005). The efficacy and safty of exogenous melatonin for primary sleep disorders a meta-analysis. Journal of General Internal Medicine 20 (12).1151-1158.
4. Murch, S.J., KrishnaRaj, S., &Saxena, P.K. (2000). Tryptophan is a precursor for melatonin and serotonin biosynthesis in vitro regenerated St. Jhon´swort (Hypericum
5. Reiter,R.J., Calvo, J.R., Karbownik,M., Qi,W., &Tan,D.X., (chromatography–mass spectrometry Original Research Article Journal of Chromatography A, 844(1–2), 295-305.

   2000). Melatonin and it relation to the immune system and inflammation. Annals of the New York Academy of Sciences, 917, 373-386.
6. Tan, D.X., Hardeland, R., Manchester, L.C., et al. (2003). Mechanistic and comparative studies of melatonin and classic antioxidants in therms of their interactions with the ABTS cation radical. Journal of Pineal Research, 34(4), 249-259.
7. Siu, A.W., Maldonado, M., Sanchez-Hidalgo, M., Tan, D.X., & Reiter, R.J. (2006). Protective effect of melatonin in experimental free radical-related ocular diseases.Journal of Pineal Research.40(2).101-109.
8. Hardeland, R., Pandi-Perumal, S.R., &Cardinali, D.P. (2006). Melatonin. The International Journal of Biochemistry & Cell Biology, 38(3), 313-316.
9. Tan, D.X., Manchester, C., Di Mascio, P., Martinez, G.R., Prado, F.M., & Reiter, R.J. (2007). Novel rhythms of N1-acetyl-N2-formyl 5methoxykynuramine and its precursor melatonin in water hyacinth: Importance for phytoremediation. The FASEB Journal, 21, 1724-1728.
10. De la Puerta, C., Carrascosa-Salmoral, M.P., García-Luna, P.P., Lardone, P.J., Herrera, J.L., Fernandez- Montesinos, R., Guerrero, J.M., Pozo, D., (2007). Melatonin is a photochemical in olive oil. Food chemistry 104, 609-612.
11. Reiter, R. J.; Manchester, L. C.; Tan, D. X. Melatonin in walnuts (2005). Influence on levels of melatonin and total antioxidant capacity of blood. Nutrition, 21 (9), 920-924.
12. Iriti, M.; Rossoni, M.; Faoro, F. (2006). Melatonin content in grape: myth or panacea? Journal of the Science of Food and Agriculture, 86, 1432-1438.
13. Cao, J; Murch, S.J.; O'brien, R; Saxena, P. K. (2006). Rapid method for accurate analysis of melatonin, serotonin, and auxin in plan samples using liquid chromatography-tandem mass spectrometry. J. Chromatography A, 1134 (1-2), 333-337.
14. Ali, I.; Aboul-Enein, H.Y.; Gupta, V.K. (2007). Analysis of melatonin in dosage formulation by capillary electrophoresis. Journal of Liquid Chromatography and Related Technologies, 30 (4), 545-556.
15. Kolar, J.; Machackova, I. (2005). Melatonin occurrence in higher plants: occurrence and possible functions. Journal of Pineal Research, 39, 333-341.
16. René J.J Vreuls, Arnold van der Heijden, UdoA.Th Brinkman, Mohamed Adahchour (1999). Trace-level determination of polar flavour compounds in butter by solid-phase extraction and gas
17. Wang. Z.H. J. Dou, D. Macura, T.D. Durance, S. Nakai (1997). Solid phase extraction for GC analysis of beany flavours in soymilk Original Research Article Food Research International, Volume 30, Issue 7, August 1997, Pages 503-511.
18. Yolanda Picó, Mónica Fernández, MariaJose Ruiz, Guillermina Font (2007).Current trends in solid-phase-based extraction techniques for the determination of pesticides in food and environment.Journal of Biochemical and Biophysical Methods, 70(2), 117-131.
19. Insa. S, E. Anticó, V.Ferreira (2005).Highly selective solid phase extraction and large volume injection for the robust gas chromatography–mass spectrometric analysis of TCA and TBA in wines Original Research Article Journal of Chromatography A, 1089 (1–2), 235-242.
20. Albero Beatriz, Consuelo Sánchez-Brunete, José L. Tadeo(2005). Multiresidue determination of pesticides in juice by solid-phase extraction and gas chromatography–mass spectrometry Original Research Article Talanta, 66 (4),917-924.
21. SibelTopuz, GülÖzhan, BuketAlpertunga (2005).Simultaneous determination of various pesticides in fruit juices by HPLC-DAD Original Research Article Food Control, 16(1), 87-92.
22. Khrolenko Maxim, PawełDżygiel, PiotrWieczorek (2002). Combination of supported liquid membrane and solid phase extraction for sample pretreatment of triazine herbicides in juice prior to capillary electrophoresis determination Original Research Article Journal of Chromatography A, 975(1), 219-227
23. Albero. B, C Sánchez-Brunete, J.L Tadeo (2003). Determination of endosulfan isomers and endosulfan sulfate in tomato juice by matrix solid-phase dispersion and gas chromatography Original Research Article
    Journal of Chromatography A, 1007(1–2),137-143
24. Gergely A Csiktusnádi Kiss, Esther Forgács, TiborCserháti, Manuel Candeias, Louis Vilas-Boas, Rosario Bronze, Isabel Spranger (2000). Solid-phase extraction and high-performance liquid chromatographic separation of pigments of red wines.Journal of Chromatography A, 889(1–2), 51-57
25. Guofang Chen, YushuHuo, Dun-Xian Tan, Zhen Liang, Weibing Zhang, Yukui Zhang (2003). Melatonin in Chinese medicinal herbs. 73, 19-26.