# Methods of purification of raw polyphenol extract for chromatographic analysis

## Metody oczyszczania surowego ekstraktu polifenoli do analizy chromatograficznej

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**Abstrakt**

W artykule opisano metody oczyszczania ekstraktów polifenolowych zarówno w preparatach otrzymywanych ze świeżych owoców i warzyw, jak i z produktów przetworzonych. W pracy opisano metodę oznaczania stężenia substancji polifenolowych za pomocą technik chromatograficznych (HPLC, HSCCC) z uwzględnieniem poszczególnych metod oczyszczania preparatów.

**Abstract**

The article describes the methods of purifying polyphenolic extracts both in preparations obtained from fresh fruit and vegetables and from processed products. The paper describes the method of determining the concentration of polyphenolic substances by means of chromatographic techniques (HPLC, HSCCC).

*Słowa kluczowe:* polifenole, metody oczyszczania ekstraktów polifenoli

*Keywords:* polyphenols, methods of purification of polyphenolic extracts

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## 1. Introduction

In recent years, considerable research has been conducted on polyphenols in foods, focusing on their bioavailability, metabolism and biological effects. This was driven by results of epidemiological studies which showed that diets rich in plant foods are associated with decreased incidence of cancer and heart diseases. Plants are rich in polyphenols, and the extraction and enrichment of these bioactive compounds from fruit is of increasing academic and commercial interest[1]. Polyphenols are organic compounds from the group of phenols, i.e. alcohols, that have a hydroxyl group connected to the aromatic ring. The name of the polyphenolic compounds indicates that there must be at least two such hydroxyl groups. These compounds occur naturally and are important components of plants, and contribute to colour, aroma and taste, as well as having many other functions. Green tea, grapes, blueberries, olive oil, nuts, beans, cocoa, yerba mate and some vegetables (especially legumes) and fruits are rich in polyphenols. Polyphenols in fruit extracts have an important value in food and medicine, but also, a large amount of polyphenols is found in by-products of fruit processing, consisting of peel and seeds, which can be used in the circular economy. For example, a significant amount of polyphenols, known for their antioxidant and antimicrobial properties can be extracted from pomegranate peel [2, 3]. The extraction of polyphenols from secondary resources is the subject of much research [4, 5] and has a wide range of applications presenting a great potential on the Green Deal implementation. The association of polyphenol extracts with other natural products extracted from secondary resources, such as protein extracts, expands the range of applications to new pharmaceuticals, cosmetics and even agrochemicals, along with those developed for food and medicine. In this context, the development of methods for the separation and quantification of compounds from extracts containing polyphenols is a challenge with a major impact in the future. From this perspective,

chromatography is one of the most important and dynamic methods for separation, identification, and quantification of phenolic compounds [6]. This paper brings innovative approaches in the development and adaptation of analytical protocols to expand the chromatographic methods for the complex extracts polyphenols study.

## 2. High performance liquid chromatography (HPLC) – sample preparaion for chromatography HPLC technique

Amongst the different methods available, HPLC is preferred for the separation and quantification of polyphenolics in foodstuffs extracts. Nevertheless, HPLC methods present limitations especially in complex matrix, such as crude plant extracts and environmental samples. Thus, an initial preconcentration and purification of the polyphenols from complex matrix is crucial prior to the instrumental analysis by HPLC. The aim of preconcentration is to simplify the chromatograms obtained so that they can be reliably identified and quantified. The purification stage is the critical part of a method, the removal of potential interfering components varies according to the vegetal matrix to be analysed. The procedure includes liquid– liquid partitioning with a immiscible solvent and open column chromatography on Sephadex LH-20, polyamide, Amberlite, prep-HPLC and solid phase extraction (SPE) using commercially available cartridges[7].

## 3. Solid Phase Extraction

Solid Phase Extraction (SPE) is one of the simplest but most effective and versatile sample preparation methods. Therefore, the aim of Michalkiewicz, A., Biesaga, M., & Pyrzynska, K. (2008), work was to study in more detail the SPE process of phenolic acids (such as gallic, p-HBA, p-coumaric, vanillic, coffee and syringic acid) and some flavonols (rutin, quercetin and kaempferol) in honey. After selecting the appropriate chromatographic and detection conditions, most of the

steps of the SPE procedure affecting recovery (amount of sorbent, composition of elution and washing liquids) were tested using standard aqueous samples. The selectivity of the procedure was checked in enriched honey samples, and the operation of the method was tested on real honey samples of various botanical origins[8].

The SPE cartridges used were Bond Elut octadecyl C18 (500 mg) from Varian, Oasis HLB (200 mg) from Waters and Strata-X (30 mg) obtained from Phenomenex. They were conditioned by washing with 4 ml of methanol and 2 ml of deionized water. Oasis powder was also used to prepare the columns. Amberlite XAD-2 (Aldrich) was first regenerated with a 2 molar L-1HCl solution and then conditioned with methanol. Honey samples (20 g) were mixed with 100 mL of deionised water adjusted to pH 2 with HCl and stirred in a magnetic stirrer for 15 min. The fluid samples were then filtered through cottonwool to remove the solid particles. Extraction was performer with the vacuum station from Varian. The filtrate was passed through an appropriate cartridge, which was then washed with 50 ml of acidified water to remove all sugars and other polar constituents of honey. The adsorbed compounds were eluted with methanol (50 ml). The extract was concentrated to 5 ml under reduced pressure in a rotary evaporator at 40°C, filtered through a 0.45-m membrane filter and injected into HPLC system. The entire extraction procedure was repeated simultaneously at least three times for each kind of tested sorbent[8].

## 4. Macroporous resins

Polyphenols can usually be purified by adsorption–desorption processes by using highly efficient sorbents, of which C18 and highly crosslinked styrene–divinylbenzene (S–DVB) copolymers are very popular[2]. The researcher objective

of the reaserch works was thus to assess the use of macroporous resins for the concentration of polyphenols from leaves[9].

Food grade macroporous resins Amberlite XAD-7 and XAD-16 and Relite EXA-90 and EXA-118 were used as adsorbents. Thenecessary preconditioning of the adsorbents was realized by anextensive wash with abundant distilled water to remove saltsand impurities, and was then followed by a drying at 70°C for 24 h and posterior cooling in a desiccator. The dried resin was immersed in ethanol for 12 h. The ethanol was then replaced by distilled water through washing. From the IEE (I.edulis extract – leaves extract), different solutions (20 up to 6000 mg GAE/l)were prepared in sodium citrate/citric acid buffer (pH 3.50). In order to construct the sorption isotherms, 10 ml ofthese buffered concentrations of IEE were put in contact with 50 mg of resin, inside amber glass bottles of 50 ml, and were saturated with gaseous nitrogen. These bottles were submittedto planetary agitation at 200 rpm during 2 h and the tempera-ture was kept at 25°C[9].

In other studies, the preconcentration was accomplished by the adsorption–desorption method with a styrene–divinylbenzene resin (XAD-4) or XAD-16 and the results demonstrated that both resins are capable of successfully adsorbing polyphenols. However, in most of the works for determining polyphenols, solid phase extraction (SPE) was used for purification, and the analytes were usually eluted with methanol, ethanol or their aqueous form[7].

## 5. $CO_2$ purification process

The Mauro Bleve, Loredana Ciurlia, Elisa Erroi,Giulia Lionetto, Luigia, Longo, Leonardo Rescio, Trifone Schettino, Giuseppe Vasapollo work describes a new and innovative method for the purification of anthocyanins from grape skin extracts as liquid matrix (LM), by using carbon dioxide ($CO_2$). The LM was obtained by extraction of anthocyanins from grape skins using a water/ethyl alcohol solution

acidified with 0.2% trifluoroacetic acid (TFA). The $CO_2$ purification process produced a desired fraction (F1) containing pure anthocyanins, and a second fraction (F2) containing ethyl alcohol and other components of the grape skins. An yield of anthocyanin of about 85%, with respect to the starting overall anthocyanin content, was obtained under the following optimized process conditions: pressure 100–130 bar; temperature 30–40 °C; pH of LM 2–4; percentage of ethyl alcohol in the LM 25–30%; $CO_2$ flow rate 25–50 ml/min; LM flow/$CO_2$ flow ratio 3–10%. In the papere is also demonstrated that the purification process allowed to eliminate ethyl alcohol from the anthocyanin extract without any thermal or chemical degradation, obtaining a high anthocyanin value product which maintains its anthocyanin content and antioxidant activity unchanged[10].

## 6. HSCCC (High Speed Counter Current Chromatography)

High speed counter current chromatography (HSCCC) utilizes a liquid stationary-phase and, hence, does not suffer from irretrievable adsorption associated with conventional chromatography procedures. It is a good alternative for occur traditonally chromatograhy techniques because of its speedier and economically viable separation, ease of scaling-up, ability for coupling with other analytical instruments for establishing on-line hyphenated systems, elevated sample-load capacity, truncated solvent consumption, absence of irretrievable sample adsorption, and availability of a diverse range of solvent-systems and elution modes [11].

Apple pomace, a by-product in the processing of apple juice, was investigated as a potential source of polyphenols. Two methods of separation and purification of polyphenols from apple pomace extract were established by combination of gel chromatography with high-speed counter-current chromatography (HSCCC) and solvent extraction with HSCCC, respectively. The optimal separation was

performed on a Sephadex LH-20 column using gradient aqueous ethanol as eluting solvent from 0% to 100% in increments of 10%. HPLC analysis indicated that main polyphenols existed in fractions eluted between 40% and 50% aqueous ethanol. The fractions of interest from column were separated by HSCCC with the solvent system hexane–ethyl acetate–1% aqueous acetic acid (0.5:9.5:10, v/v/v). Ethyl acetate fractionation of the apple pomace extract followed by direct HSCCC separation by the same solvent system in the volume ratio of 1:9:10 also produced a good separation of the main polyphenols of interest. Six high-purity polyphenols were achieved tentatively and identified by HPLC/MS: chlorogenic acid (1, m/z 354), quercetin-3-glucoside/quercetin-3-glacaside (2, m/z 464), quercetin-3-xyloside (3, m/z 434), phloridzin (4, m/z 436), quercetin-3-arabinoside (5, m/z 434), and quercetin-3-rhamnoside (6, m/z 448)[6]. The HPCCC technique is a non-standard and expensive technique. In addition, it is characterized by a time-consuming optimization process, the lack of standard solvents and the fact that it is possible to work with two-phase systems[12].

## 7. What is the most effective method of purifying raw polyphenol extracts?

The answer is - it depends on the method of preparation of polyphenol solutions and the type of sample (fresh fruit, pomace, peel, fruit or vegetable waste) and the sensitivity of the chromatographic technique used and the last what sort of compunds are obtaind within pufification for example - anthocyanins.

- Sort of sample - fresh fruits (Pica, Sutil and Genova pulp)

Fresh citrus fruits were washed, the peels manually separated and the pulp deseeded. Each peel and pulp-juice (100 g) were separately homogenized in a blender with 100 mL of 0.1% HCl in MeOH and extracted thrice for one hour each time under dark using an ultrasonic bath. The extracts were combined, filtered and the solvent removed *in vacuo* (40 °C). The remaining aqueous partitions were

separately loaded in an Amberlite XAD-7 column (5 cm × 15 cm) and rinsed with 100 ml ultrapure water. The phenolic compounds present in each partition were then eluted with 100 mL of 0.1% HCl in MeOH obtaining 0.62, 0.91 and 0.63 g of Pica, Sutil and Genova pulp extracts, respectively, and 0.76, 0.69 and 0.54 g of Pica, Sutil and Genova peel extracts, respectively. The extracts (aprox. 2 mg) were redissolved in 2 ml 0.1% HCl in MeOH, filtered through a 0.45 μm micropore membrane (PTFE, Waters) before use and 10 μL were injected into the HPLC instrument for analysis[14].

- Anthocyanins – dry products

The main goal of this work was to develop a potentially scalable extraction procedure that gave anthocyanins in a reasonably concentrated form and in the absence of any co-extractants (e.g. free sugars). Dried black currant epicarp (30 g) was immersed in 600 ml water acidified with 0.01% *v*/*v* conc. HCl and stirred gently by magnetic follower at room temperature for 2 hours. The plant material was filtered off and the resulting aqueous extract loaded on to an Amberlite XAD-7HP resin (60 g) until the eluent was almost colorless. The resin was then washed with acidified water (0.01% *v*/*v* conc. HCl, 1l) before eluting the polyphenols with acidified ethanol (0.01% *v*/*v* conc. HCl). The collected ethanol fractionwere combined and concentrated under vacuum on a rotary evaporator, and then subjected to high vacuum to remove trace solvent, yielding a dark violet amorphous solid (660 mg, yield 2.2%), which could be powdered by grinding. The dried blackcurrant extract (500 mg) was then dissolved in acidified water (50 mL, 0.1% *v*/*v* conc. HCl) and partitioned against isopropyl acetate (1 × 70 mL) and ethyl acetate (3 × 50 mL) in sequential manner. The organic layers were dried under reduced pressure to give isopropyl acetate extract (yellow amorphous solid, 68.5 mg) and ethyl acetate extract (yellow amorphous solid, 33 mg), where as aqueous layer was freeze-dried to afford a red amorphous solid (399 mg)[15].

- novelty enzymatic technique

A plant polyphenol is prepared by ultrasonic or heating extracting plant raw material under the action of complex enzyme and using solvent, collecting extracting solution, clarifying by adding natural clarifying agent, filtering and collecting filtrate, purifying filtrate by adsorption resin column chromatography, ultra-filtering purified filtrate, nano-filtering, and removing solvent[16].

## 8. Conclusions

From 85 of a total of 99 peer-reviewed articles analyzed, data on solubility-based separations (liquid–liquid extraction and solutes precipitation), adsorption-based separation (including solid-phase extraction, SPE), particle-size-based separation (molecular-weight cut-off dialysis, MWCOD; and ultrafiltration, UF) and molecular-charge-based separation (capillary electrophoresis, CE)[13]. Based on a review of the literature on the methods of isolation and identification of polyphenols, it can be concluded that the key step in the analysis of this group of compounds is the appropriate sample preparation and selection of the appropriate isolation method. This paper presents various variants of extraction, purification and concentration of analytes. The literature shows that the most frequently used analytical technique for the determination of phenolic acids is high performance liquid chromatography (HPLC) [17].

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