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Development of a green extraction procedure with super/subcritical fluids to produce extracts enriched in oleuropein from olive leaves

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ABSTRACT

The extraction of olive leaves was examined by means of selective and environmentally friendly technologies using solvents in super/subcritical state. The goal was to apply different, yet complementary techniques, based on the fractionation of the extract and its enrichment in oleuropein. The leaves were extracted with supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE). Non-toxic solvents were used, namely CO_2, water and ethanol. The effect of the techniques was studied upon the extracts' yield, oleuropein content and scavenging radical activity. The serial combination of SC-CO_2 modified by 5% ethanol and subcritical water afforded high extract yield (44.1%), high recovery of oleuropein (4.6%) and good antioxidant activity. It was suggested that the removal of non-polar compounds with SC-CO_2 resulted in the enrichment of the residue in oleuropein, which was then extracted with PLE by subcritical water. The proposed approach provides the base for the establishment of a productive "green" extraction.

1. Introduction

Olive leaves gather the interest of the scientific community and the industries worldwide as their health promoting benefits are constantly being shown by an ever-increasing number of scientific data [1]. They are considered byproducts of olive farming, one of the most important activities in the Mediterranean region, representing almost 10% of the total weight of materials arriving to the olive mill. Research into olive leaves has revealed that their health properties are attributed to a group of secondary metabolites they contain, namely biophenols that display a wealth of both structural variety and diversity of important activities [2]. They contain phenolic compounds including flavones (luteolin-7-glucoside, apigenin-7-glucoside, diosmetin-7-glucoside, luteolin, and diosmetin), flavonols (rutin), flavan-3-ols (catechin), substituted phenols (tyrosol, hydroxytyrosol, vanillin, vanillic acid, and caffeic acid) and secoiridoids (oleuropein) [3]. Their content in these high added value compounds is behind the potent radical scavenging power that olive leaves' extracts exhibit. Among the bioactive compounds of olive leaves, oleuropein presents very interesting pharmacological activities. Studies have shown that oleuropein exhibits anti-ischemic, antioxidative, hypolipidemic, antiviral, antimicrobial, antiatherogenic, cardioprotective, antihypertensive and anti-inflammatory properties [4–7].

The extraction of pharmacologically active compounds from herbal plants is one of the most critical steps in natural products research. Many parameters affect the extraction procedure. Briefly, the efficiency of the extraction depends on the nature of the sample matrix, the analyte to be extracted and the location of the analyte within the matrix [2,8]. Depending on the compounds to be extracted olive leaves have been treated by means of stirring, heating and Soxhlet with different types of solvents (hexane, water, ethanol and methanol). The development of new methods and techniques that respect stringent environmental regulations are at the focus of science [2,9]. Olive leaves have been manipulated with supercritical CO_2 for the extraction of tocopherols [10], supercritical CO_2 with co-solvent ethanol for waxes, hydrocarbons, squalene, β-carotene, triglycerides, α-tocopherol, β-sitosterol and alcohols [11], supercritical CO_2 with methanol for carboxylic acids and phenols [12] and water/ethanol/citric acid mixtures for phenolics [13]. For the extraction of oleuropein innovative methods have emerged that are mainly based on the usage of polar solvents and accelerated energies. Superheated liquid extraction through a static-dynamic approach [14], ultrasound-assisted [15] and microwave-assisted [16] extraction with ethanol–water have been proposed, along with its separation and purification with the use of macroporous resins [17]. The removal of chlorophylls and lipids is considered necessary for the acquisition of extracts with desirable compounds. Some

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authors have used toxic solvents, like dichloromethane for this initial process [13].

Supercritical fluid extraction (SFE) offers special advantages over conventional liquid-solvent extraction such as increased selectivity, expeditiousness, automaticity and environmental safety, in addition to dramatically decreased use of organic solvents that result in safe extracts without toxic contaminants. SFE with carbon dioxide (SC-CO_2) is the most favored method for isolation of mainly apolar or lipophilic natural compounds from spices and agricultural by-products. As a result, its application on natural products is constantly increasing [18,19]. Although SFE has been elaborated for the extraction of oleuropein, it requires high percentages of co-solvent (mainly methanol and ethanol). The effect of pressure, temperature, and type of co-solvent on the amount of extract and oleuropein has also been studied [12,20].

Pressurized liquid extraction (PLE) often named as accelerated solvent extraction (ASE) uses elevated temperatures to increase the kinetics of the extraction process while applying high pressures to maintain the solvents in their liquid state. The importance of enabling the use of solvents at temperatures above their atmospheric boiling point is the enhanced solubility and mass transfer properties. It is characterized by reduced operational cost and controlled extraction conditions, which provides consistent qualitative and quantitative composition of the extract [8].

The aim of the present study was to establish a clean, fast, highly sensitive and viable method for production of extracts with high oleuropein content taking advantage of the selectivity of SC-CO_2 for non-polar compounds and the use of polar solvents in their liquid or subcritical state in PLE. In this respect, contemporary environmentally friendly technologies were assayed for their potential in effective production of high yield extracts, rich in oleuropein, with high antioxidant capacity from olive leaf matrix, thus making easier and more accurate their subsequent application in the fields of medicine, cosmetics and food.

2. Materials and methods

2.1. Solvents and reagents

The 2,2-diphenyl-1-picryl-hydrazyl (DPPH) reagent was obtained from Sigma–Aldrich (USA). Carbon dioxide (for the extraction procedure) was supplied by Air-Liquid Hellas in cylinders and the absolute ethanol (for DPPH tests and supercritical CO_2 extraction apparatus) was obtained by Carlo Erba Reagents. Methanol (HPLC grade) was obtained from Merck KGaA, Darmstadt and acetic acid 100% glacial from Riedel-de Haën. HPLC-grade water was obtained by double-distillation and purification with a Labconco Water Pro PS polishing station (Labconco Corporation, Kansas City, MSU).

2.2. Plant material

Olea europaea (variety Koroneiki) leaves were collected in 2009 in the region of Attica, dried in a well ventilated shady place and subsequently stored. Before the extraction the leaves were grinded using an AllenWest type SCIS grinder with a sieve of 3 mm.

2.3. Supercritical fluid extraction (SFE)

The supercritical fluid extraction was performed in a pilot-scale apparatus (SFE 1-2 No. 4218, SEPAREX), which is designed to allow the study of a wide range of conditions. It consists of a CO_2 tank, a liquid CO_2 pump (that can deliver up to 10 kg/h), 2 extraction vessels (1 L and 2 L, respectively) which are both connected directly and parallel between them, 3 separators (with 200 mL capacity each), a co-solvent pump (with 40 mL/min maximum flow rate) and a cooling system. The extractions were performed within a stainless steel basket placed in a 1 L tubular extractor. The pressure in the separators compartment was kept constantly at 5 MPa. The extraction temperature was 50 °C and the separation temperature was 55 °C. The extraction was dynamic with continuous recycling of the solvent. For more details on the extraction parameters see tables in the results section. Finally, the extracts were evaporated to dryness using a rotary evaporator (Buchi Rotavapor R-200) at 45 °C and subsequently submitted to lyophilisation.

2.4. Pressurized liquid extraction (PLE)

A Dionex Accelerated solvent extraction (ASE) 300 System (Dionex, Sunnyvale, CA) with 100 mL stainless steel vessels was used for the pressurized liquid extraction. Specifically 6.0 g of grinded olive leaves powder were placed each time into the tubular extraction cells. These were then placed into the carousel and the samples were extracted under the specified conditions. The pressure applied was kept constantly at 10.34 MPa. The procedure was static. For more details on the extraction parameters see tables in the results section. Finally, the extracts were evaporated to dryness using a rotary evaporator (Buchi Rotavapor R-200) at 45 °C and subsequently submitted to lyophilisation.

2.5. HPLC analysis

The quantitative determination of oleuropein was performed in a HPLC-DAD system: Thermo Finnigan HPLC system (Thermo Finnigan, San Jose, CA) coupled with a Spectral System UV6000LP PDA detector. A two solvent gradient method was used: A. H_2O + 1% acetic acid and B. MeOH. The flow rate was set at 1 mL/min and the following elution program was applied: 0–2 min linear gradient to 5% B; 2–10 min linear gradient to 25% B; 10–20 min linear gradient to 40% B; 20–30 min linear gradient to 50% B; 30–34 min 50% B isocratic; 34–45 min linear gradient to 90% B; 45–50 min 90% B isocratic; 50–60 min linear gradient to 100% B; 60–65 min 100% B isocratic. Available standard solution of oleuropein was prepared in 50% aqueous/methanol and run under the same conditions as the samples. The analysis was performed at 25 °C and the injection volume was 20 μL. The detection was done at 248 nm and the column used was Supelco Analytical Discovery HS C18 (25 cm × 4.6 mm i.d., 5.0 μm).

2.6. TLC analysis

Merck silica gel 60 F254 (Art.5554). Detection: UV-light, spray reagent (vanillin–H_2SO_4 on silica gel).

2.7. Antioxidant activity – DPPH method

DPPH (2,2-diphenyl-1-picrylhydrazyl) is a simple and inexpensive method to measure the antioxidant capacity of extracts. It is a free radical that has the ability to be reduced to 2,2-diphenyl-1-picrylhydrazine in the presence of antioxidant compounds. The assay was based on the publication of Lee et al. [21]. The procedure involves dissolving 12.4 mg of DPPH in 100 mL of ethanol. Then 10 μL of the solution is added to 190 μL of an extract solution (dissolved in DMSO) and the mixture is left for incubation (at 37 °C) for 30 min in the dark and the absorbance is measured at a wavelength of 517 nm. All the samples were tested in a final concentration of 200 μg/mL and active were considered the extracts that reduced the free radical at a percentage >80%. All the experiments were performed in triplicate. The whole assay took place in the 96 well plate

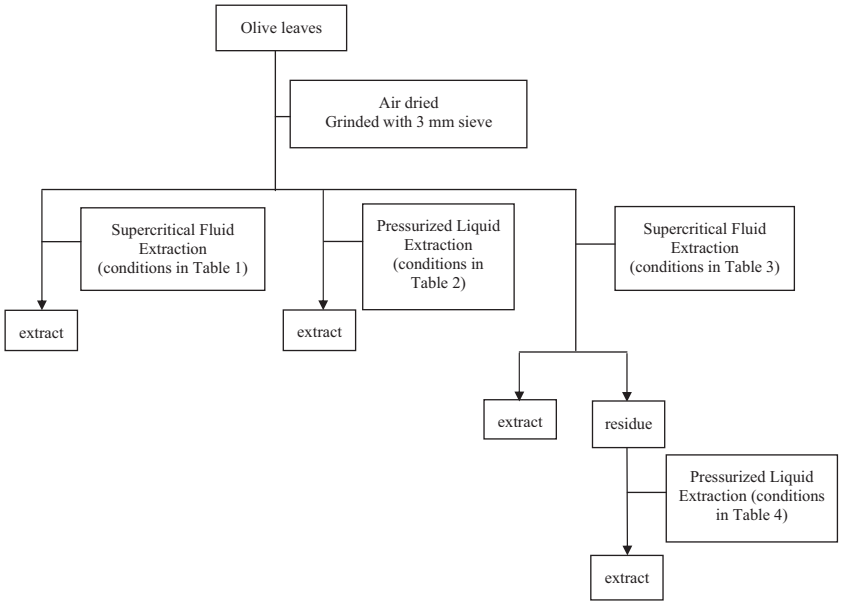

Fig. 1. Schematic representation of experimental procedure.

using an Infinite m200 pro TECAN reader (Tecan Group, Männedorf Switzerland).

3. Results and discussion

Dried and pulverized olive leaves were treated with methodologies developed on SFE and PLE having as a goal to obtain high yield extracts, rich in oleuropein that would exhibit high radical scavenging activity. State-of-the art super/subcritical fluid extractions with CO_2, water and ethanol were used, exploiting the unique features of each technique (Fig. 1).

As a first approach olive leaves (275.0 g) were extracted with SC-CO_2 modified with ethanol in order to investigate the amount of oleuropein that would be possible to recover and the required parameters, mainly concerning the necessary amount of co-solvent. The fact that CO_2 is considered an apolar solvent rendered necessary the addition of ethanol as co-solvent. Ethanol was selected because of its low toxicity compared with other options. Thereby, the applied pressure was 30 MPa, in order to increase fluid density and solvating power of CO_2 and the percentage of ethanol initially reached 5%. Under these conditions the yield of the dry extract was 14.7% and no oleuropein was detected. Following this, pressure remained at a high level and the percentage of ethanol was increased to 20%. These parameters had as a consequence the acquisition of 46.75 g of dry extract (17.0% yield) (Table 1). In addition, the extract was analyzed for oleuropein (Fig. 2). The dry extract contained 30.0% oleuropein equivalent to 5.1% recovery (g/100 g of olive leaves). However, the process required high amounts of CO_2 and co-solvent leading to a costly procedure.

PLE was elaborated in order to comparatively study its effectiveness in the extraction process of the polar constituents from olive leaves. In order to maximize the recovery of oleuropein, water and ethanol were utilized. Ethanol (at 40 °C), water (at 50 °C), subcritical ethanol (at 115 °C), subcritical water (at 150 °C) and a water/ethanol 40:60 mixture (at 50 °C) were used. 6.0 g of sieved olive leaves were used in each case. The parameters of the extraction are depicted in Table 2. It was observed that the yields of the extracts were high and the parameters employed allowed the extraction of polar compounds. The highest yield (41.5%) was achieved with subcritical water. The application of high

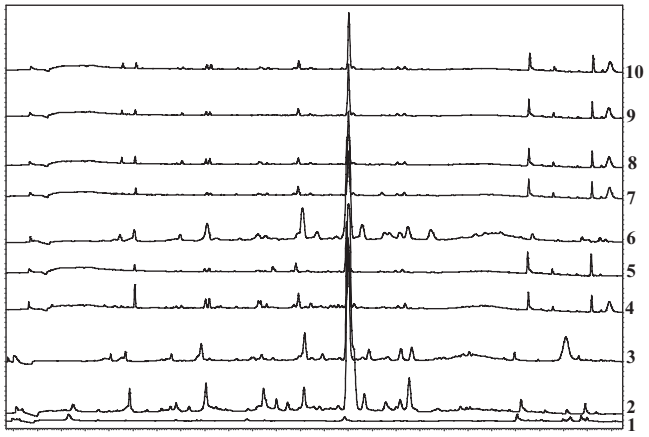

Fig. 2. Overlay of HPLC chromatograms of extracts obtained from olive leaves: 1. SFE with 5% ethanol, 2. SFE with 20% ethanol, 3. PLE with ethanol 100% at 40 °C, 4. PLE with water 100% at 50 °C, 5. PLE with water/ethanol 40:60 at 50 °C, 6. PLE with subcritical ethanol 100% at 115 °C, 7. PLE with subcritical water 100% at 150 °C, 8. PLE (after SFE) with water 100% at 50 °C, 9. PLE (after SFE) with water/ethanol 40:60 at 50 °C and 10. PLE (after SFE) with subcritical water 100% at 150 °C. Major peak represents oleuropein.

Table 1
Conditions of SFE for the extraction of oleuropein and obtained results.

Ratio[a]	Flow rate (kg/h)	P (MPa) in extractor	Co-solvent (wt%)	Dry extract (g)	Yield (w/w%)	Oleuropein (%)[b]	Recovery (%)[c]	DPPH (μg/mL)
120	9.6	30	5%	40.42	14.7	0.0	0.0	752.1
290	9.6	30	20%	46.75	17.0	30.0	5.1	113.9

[a] Solvent-to-feed ratio.
[b] (%) oleuropein in the dry extract.
[c] (%) oleuropein in olive leaves.

Table 2
Conditions of PLE for the extraction of oleuropein and obtained results.

T (°C)	Heat (min)	Pre-heat (min)	Solvent	Static time (min)	Cycles	Dry extract (g)	Yield (w/w%)	Oleuropein (%)[a]	Recovery (%)[b]	DPPH (μg/mL)
40	5	1	EtOH	10	2	1.57	26.2	14.5	3.8	123.4
50	5	1	H_2O	10	2	0.93	15.5	10.7	1.7	128.0
50	5	1	H_2O/EtOH (40:60)	10	2	1.60	26.7	7.3	1.9	124.0
115	5	1	EtOH	10	2	1.58	26.4	16.4	4.3	134.7
150	5	1	H_2O	10	2	2.49	41.5	8.3	3.4	132.5

[a] (%) oleuropein in the dry extract.
[b] (%) oleuropein in olive leaves.

temperature during the extraction causes the decrease of the dielectric constant (polarity) of the water, resulting in parallel in a steady decrease in viscosity and surface tension, as well as in faster diffusivity characteristics. Qualitative characterization of the extracts by RP-HPLC showed that the (%) content and recovery of oleuropein were higher at the extract obtained with subcritical ethanol (16.4% and 4.3%, respectively). However, these percentages did not differ much from the ones obtained with subcritical water (8.3% and 3.4%, respectively), thus the use of water was also considered advantageous (Fig. 2). Although, ethanol proved again an effective extractant for oleuropein, a procedure performed with 100% ethanol is not easily applicable due to its high-cost.

After experimentation with both techniques separately, their combination was investigated based on the competence of each technique. The preference of SC-CO_2 for the extraction of the undesirable apolar and lipophilic substances (fatty substances, waxes and chlorophylls) from the leaf matrix led to its elaboration for defatting of the initial material, while PLE with polar solvents was used for the isolation of the polar compounds of interest. The addition of co-solvent, specifically ethanol at percentage high enough to assist in the removal of lipophilic compounds but not permitting the extraction of oleuropein was considered essential. Initially, olive leaves were placed at the SFE apparatus for extraction with CO_2. One of the most important advantages of SFE is the possibility of changing operational conditions to facilitate the extraction of specific compounds. As a consequence, in the present study, the procedure proceeded with gradual increase of the pressure in order to achieve the fractionation of the extract and the acquisition of a defatted residue. 412.4 g of tree olive leaves were placed inside the 1 L extractor of the apparatus. TLC of the extracts obtained and co-chromatography with standard compounds permitted a quick preview of their chemical content. The details and the parameters of the trial are depicted in Table 3. Initially, low pressures (12, 15 MPa), accounting to low CO_2 density and solvating power, were selected in order to extract low molecular weight compounds. During this step of the extraction a pure white powder was deposited in the separators. Analysis with TLC revealed that the sample was dominated by waxes. As the extraction proceeded, the extract was enriched with a yellow, thick substance, which was soluble only in the supercritical state, and thus it was solidified on the walls of the separators. A subsequent rinse of the separators with ethanol was mandatory in order to remove them from the interior walls. Then, higher pressures were applied (20, 25 MPa), keeping the temperature constant aiming to maximize the density and thus the solvating power of CO_2 in order to remove higher molecular weight compounds. The extract had an intense yellow color and analysis indicated the presence of fatty acids and a small amount of carotenoids. Under these conditions the extract was again not completely removed from the separators and a subsequent rinse with ethanol was necessary. Since the yield of the extraction was not significantly high, a further increase of pressure, also resulting in polarity modification was applied in the system in order to improve the yield and the selectivity of the process. Pressure of 30 MPa was applied and the mobile phase was modified with ethanol (1–5%). The addition of the co-solvent was again gradual and cautious since the point that the majority of the lipophilic compounds along with chlorophylls would be removed from the residue and oleuropein would be extracted, should be identified. The extract had a green color indicating the presence of chlorophylls. TLC analysis revealed that terpenoids were also obtained and more specifically oleanolic acid, maslenic acid and β-sitosterol. Sequentially the amount of co-solvent increased to 10%. Extract yield increased with co-solvent administration, compared to the previous samples. Of interest was the isolation of hydroxytyrosol and oleuropein only in traces. It was decided that the extraction should stop at this point and the residue was placed into the PLE apparatus in order to continue with the extraction of the polar compounds. Three different methodologies were applied, focused on maximizing the recovery of oleuropein:water (at 50 °C), subcritical water (at 150 °C) and a water/ethanol 40:60 mixture (at 50 °C). 6.0 g of the SFE's residue were used in each case while the parameters are depicted in Table 4. It was observed that the residue of the SC-CO_2, which was processed afterwards with PLE, gave really high yields (33.5–44.1%) of dry extracts. These results were due to the removal of the fatty components from the leaf matrix. Oleuropein was detected at t_R = 30.4 min as the major peak in all extracts analyzed. The SFE residue extracted with subcritical water gave the highest yield (44.1%) and recovery of oleuropein (4.6%) (Fig. 2). Apart from these, the removal of non polar compounds achieved with SFE and the decreased amount of sugars obtained with subcritical water gave to the final extracts additional desirable features.

Table 3
Conditions of SFE for the extraction of lipophilic compounds and obtained results.

Extractor					
Ratio[a]	Flow rate (kg/h)	P (MPa)	Co-solvent (wt%)	Dry extract (g)	Yield (w/w%)
45	5.0	12	–	4.02	0.97
70	5.0	15	–	0.06	0.01
100	5.0	20	–	0.11	0.03
130	5.0	25	–	1.18	0.29
160	5.0	30	–	0.08	0.02
350	5.0	30	1	6.00	1.45
520	5.0	30	3	8.62	2.09
610	5.0	30	5	4.24	1.03
660	5.0	30	10	4.37	1.06

[a] Solvent-to-feed ratio.

The comparison of these results with the ones obtained after the application of the two techniques separately showed that the serial combination of SC-CO_2 modified by ethanol (up to 10%), with PLE especially subcritical water, can be advantageous since it offers high yield extracts with high recovery of oleuropein. SC-CO_2 modified with ethanol (20%) and PLE with ethanol (100%) can also lead to the extraction of oleuropein but a high percentage of ethanol is necessary.

Lastly the extracts were evaluated for their radical scavenging activity with the DPPH assay. The application of the DPPH test demonstrated that the extracts exhibited radical-scavenging activity and the results appear in Tables 1, 2 and 4. As observed all the

Table 4
Conditions of PLE for the extraction of oleuropein from the defatted residue (after SFE) and obtained results.

T (°C)	Heat (min)	Pre-heat (min)	Solvent	Static time (min)	Cycles	Dry extract (g)	Yield (w/w%)	Oleuropein (%)[a]	Recovery (%)[b]	DPPH (μg/mL)
50	5	1	H_2O	10	2	2.16	33.5	9.9	3.3	138.6
50	5	1	H_2O/EtOH (40:60)	10	2	2.30	35.7	9.5	3.4	145.3
150	5	1	H_2O	10	2	2.84	44.1	10.4	4.6	127.3

[a] (%) oleuropein in the dry extract.
[b] (%) oleuropein in olive leaves.

extracts showed good antioxidant activity without significant variations. The extracts obtained by SFE modified with 20% ethanol (Table 1) and PLE with 100% ethanol (40 °C) and water/ethanol 40:60 (50 °C) (Table 2) showed the best activity. It is hypothesized that the organic solvent causes the extraction of other categories of compounds like flavonoids which also contribute to the antioxidant properties. The extracts obtained from SFE (up to 10% ethanol) had no significant antioxidant activity (data not shown), something expected since they contained no antioxidant substances. Previous studies refer that the solvent type can affect the antioxidant activity of olive leaf extracts and methanol is recommended as the solvent of choice [22]. In the present study we suggest that an extraction performed under carefully investigated conditions produces extracts with high oleuropein content and thus good antioxidant activity even when it is performed with a non toxic solvent, like water.

4. Conclusions

In the present study an efficacious, simple and selective procedure for extracting olive leaves was introduced, using super/subcritical fluids. It is based on the elaboration of contemporary techniques using "green" approaches. Compared to previous studies, the present research proposes the combination of two "green" techniques that could provide the optimum extraction of the initial material by carefully selecting the conditions promoting the recovery of the desirable compounds. The removal of lipophilic compounds, which were of minor importance in the present study since they do not contribute to the antioxidant activity of the plant, succeeded with SC-CO_2, led to a residue rich in phenolic compounds, which was extracted sequentially with subcritical water in PLE. The result was high yield extracts, rich in oleuropein that exhibited high radical scavenging activity. SFE and PLE individually provide high oleuropein content, however the need for high percentages of co-solvent/solvent is not cost effective. Furthermore, PLE with water or water/ethanol provide high extraction yields but moderate recoveries of oleuropein. The present study proposes a "green" extraction procedure which affords extracts rich in oleuropein, while in parallel minimizes extraction time, uses no harsh organic solvents and eliminates problems in extraction caused by lipids and chlorophylls. Of major importance is that the extract finally obtained is characterized by desirable traits and could be used for human consumption. The information acquired from this study will be used to optimize the extraction process accordingly and make feasible the industrial implementation of the proposed method.

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