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A simple and robust LC-ESI single quadrupole MS-based method to analyze polyphenols in plant extracts using deep eutectic solvents

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ABSTRACT

Currently, the interest in polyphenols is increasing due to their significant properties in health. Polyphenols exist in a range of natural products, however their extraction as well as their characterization are important issues as they are mainly present in complex matrices. Therefore, sensitive and selective analytical methods based on liquid chromatography coupled to tandem mass spectrometry are essential. Nevertheless, access to such high-resolution techniques is quite rare. Thus, in this work we present a simple, selective and robust method based on a single-quadrupole (Q) MS technique) for the analysis of a wide range of polyphenols such as flavonoids, phenolic acids and anthocyanins.
Specifically, we present:

- A simple liquid chromatography electro-spray ionization (LC-ESI) single-quadrupole mass selective (MS) method for the analysis of 18 different polyphenols.
- Application of the method to three plant-based extracts that are derived after green extraction methods.

Specification Table

Subject area:	*Agricultural and Biological Sciences*
More specific subject area:	*Analytic and sample pre-treatment*
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Methods details

Reagents and standards

Analytical standards of the polyphenols were a kind offer from the lab of Dr. Nadjet Benaida-Debbache in Université de Bejaia, in Algeria and were obtained from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany).

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Acetonitrile (ACN), for LC-MS (CHEM-LAB NV, Zedelgeme, Belgium), Methanol (MeOH) for LC-MS (CHEM-LAB NV, Zedelgeme, Belgium) and Acetic acid (CHEM-LAB NV, Zedelgeme, Belgium) were used for mobile phase preparation for LC–MS analysis.

Polyphenols are highly antioxidant molecules thus should carefully handle and be kept in dark during the experimental process.

Sample collection and pre-treatment

Extracts from the plants *Clematis flammula* L. leaves (CFL), *Pistacia lentiscus* L. black fruits (PBF) and *Populus nigra* L. (PN) extracts were prepared according to previously published methods [1,2] and were made available by our partners. In brief, the extracts were prepared using deep eutectic solvents (DES).

Deep eutectic solvent preparation

Deep eutectic solvents used were prepared by adding hydrogen bond acceptor (HBA) "choline chloride" and hydrogen bond donor (HBD) "acetic acid" at a ratio of (1:2) (mole/mole). The resulting mixture was placed in a round-bottom flask and heated to 80 °C in a water bath with agitation until a homogeneous liquid was formed.

Extract preparation

The extraction of polyphenols using ultrasound was investigated in this study. Briefly, the powdered plant material (50 mg) of the plant powder was added to 1 mL of DES, the mixture was agitated and then immersed in the flask, and the mixture was placed in the ultrasonic water bath. After the extraction was finished, the flask was removed and cooled to room temperature before the mixture was centrifuged at 8000 g for 20 min to ensure a complete separation. The clear solution above was transferred in 15-mL tubes and collected and analyzed.

Optimization of extraction conditions

The selection of the process levels of each factor that have significant influence on the extraction of total phenolic content (TPC) from PBF has been performed. The effect of water percentage (X1) extraction temperature (X2) and extraction time (X3) were the three major factors affecting TPC (ϒ), for each variable, 3 levels coded as (+1), (−1) and center points (0). Based on the obtained data from the factorial design, the optimal extraction of compounds from PBF using DES was explored via Box–Behnken design (BBD) and response surface methodology (RSM) [1,2].

LC-ESI/MS analytics

In this study 18 different polyphenols were identified using a Liquid Chromatography system (LC, Shimadzu, Kyoto, Japan) coupled with electrospray/MS system. The LC-ESI/MS system consisted of a binary pump (LC-20AD, Shimadzu, Kyoto, Japan) and an ESI single quadrupole MS detector (LCMS2020, Shimadzu, Kyoto, Japan). The LC method was performed on the reversed phase Syncronis^{TM} C-18 column (150×4.6 mm; particle size: 5 μm) (Thermo Fisher Scientific^{TM,} Waltham, Massachusetts USA). Specifically, the method used for the determination of the polyphenols is based on previously reported methods with modifications [3–5].

The system was operated in gradient mode at a temperature of 45 °C with a flow rate of 0.8 mL/min (Table 1). According to Table 1 the mobile phase consisted of 95% ddH2O, 5% MeOH, 0.2% acetic acid (solvent A) and 50% acetonitrile (ACN) /50% ddH2O (solvent B).

Detection was performed using an ESI/MS detector. Specifically, gas temperature was set at 350 °C, nebulizing gas flow was 1.5 L/min, drying gas flow was 15 L/min, heat block was set at 450 °C, nebulizer pressure was 45 psig; Vcap was at +4000 V, the single ion mode (SIM) was set in negative mode for: m/z 285, 254.8, 125, 109, 224.05, 609, 191, 366.8, 1060.8, 481, 607, 163, 175, 484, 149.06, 169.17, 179.15 while SIM was set in positive mode for: m/z 289, with event time 0.5 s respectively. Table 2 shows the retention time of each standard along with the corresponding ratio m/z. The injection volume was set to 50uL. A mix of standards as well as alone standards ranging from 0.05 to 20ug/L were used for calibration. All standards and samples were analyzed in triplicates. In addition, three independent experiments for all the analytical steps were performed.

Method validation

The validation of the method was based on the determination of linearity, accuracy (analytical recovery), precision calculating the detection and quantification limits (LOD and LOQ) and the relative standard deviation (RSD). The correlation coefficients of the corresponding calibration graphs were used as a measure of linearity. Moreover, the limits of detection (LOD) and quantification

Table 1
LC-gradient system.

Time (min)	Solvent A (%)	Solvent B (%)
0–20	80	20
21–40	45	55
41–65	0	100
66–75	90	10

Table 2
Retention time (min) for phenolic standards.

Phenolic standards	*m/z*	Retention time (RT)(min)
Citric acid	191.0	2.7 ± 0.01
Ascorbic Acid	175.0	2.8 ± 0.01
Cinnamic acid	149.06	2.9 ± 0.01
Gallic Acid	169.17	4.4 ± 0.001
Pyrogallic Acid	125.0	5.4 ± 0.01
Catechin	289.0	12.4 ± 0.01
Caffeic Acid	179.15	16.1 ± 0.01
Coumaric Acid	163.0	24.8 ± 0.01
Tannic Acid	1060.8	26.7 ± 0.01
Conjugated Tannic Acid	224.05	30.1 ± 0.01
Catechol	109.0	32.0 ± 0.01
Rutin	609.0	32.1 ± 0.01
Diosmin	607.0	37.5 ± 0.01
cyanidin	484.0	47.1 ± 0.01
Silymarin	481.0	47.6 ± 0.01
Kaempferol	285.0	50.0 ± 0.01
Chrysin	254.8	56.9 ± 0.01
Curcumin	366.8	62.5 ± 0.01

Table 3
Method validation parameters for the LC-ESI-MS method.

Mixture of stds	Linear Regression Equation	R^2	Recovery%	LOD/LOQ (ug/L)[1]
Chrysin	y = 0.0083x-0.0018	0.97	90.2 (± 1.02)	1/5 (± 0.02)
Pyrogallic Acid	y = 0.0095x-0.0040	0.96	87.3 (± 1.12)	0.45/3 (± 0.01)
Catechol	y = 0.1385x-0.0073	0.99	90.0 (± 1.13)	1/10 (± 0.03)
C.Tannic Acid	y = 0.0357x-0.0078	0.99	88.2 (± 1.04)	1/5 (± 0.02)
Rutin	y = 0.2121x-0.00436	0.99	82.3 (± 1.01)	0.7/3.5 (± 0.01)
Citric acid	y = 0.2876x+0.0292	0.97	89.2 (± 2.11)	0.8 / 4 (± 0.01)
Curcumin	y = 0.0018x-0.0012	0.95	90.4 (± 1.87)	1/10 (± 0.03)
Tannic Acid	y = 0.2543x-0.0169	0.96	89.8 (± 1.02)	1/10 (± 0.03)
Catechin	y = 0.0076x-0.0017	0.99	92.1 (± 1.03)	0.5/3 (± 0.01)
Silymarin	y = 0.3282x-0.0643	0.96	91.2 (± 1.67)	1/3 (± 0.01)
Diosmin	y = 0.1330x+0.0511	0.93	88.3 (± 1.01)	1/10(± 0.03)
Kaempferol	y = 0.0380x-3.0008e-5	0.94	90.4 (± 1.09)	3/10 (± 0.03)
Coumaric Acid	y = 0.0319x+0.0197	0.95	87.3 (± 1.21)	1/3 (± 0.01)
Ascorbic Acid	y= −0.0015x-0.0098	0.92	86.8 (± 1.31)	1/10 (± 0.03)
cyanidin	y = 0.0328–0.0645	0.98	84.8 (± 1.18)	0.5/1 (± 0.01)
Cinnamic acid	y = 0.0062x-0.0012	0.98	90.2 (± 2.01)	1/10 (± 0.03)
Gallic Acid	y = 0.0536x+0.0116	0.95	87.2 (± 1.17)	0.5/1 (± 0.01)
Caffeic Acid	y = 0.0161x-7.4350e-4	0.98	90.1 (± 2.11)	4/10 (± 0.03)

[1] The limits of detection (LOD) and quantification (LOQ) for the polyphenols were determined at a signal-to-noise ratio (S/N) of 3:1 and 10:1, respectively.

(LOQ) for the polyphenols were determined at a signal-to-noise ratio (S/N) of 3:1 and 10:1, respectively, by injecting a series of dilute solutions [3–5].

Accuracy, evaluated as a percent recovery, was determined by spiking in the extracts with mixed calibration solutions at three concentration levels for each analyte, 0.05 ug/L, 1 ug/L, 10.0 ug/L. For each concentration, three parallel samples were prepared and analyzed.

For each compound, the recovery was calculated by the ratio between the measured amounts in the spiked extract to the corresponding target value. The recovery rate for the low concentrations was more than 80% while for higher concentration was between 82 and 92%. The reproducibility, presented as standard deviation, between 1 – 3% was satisfying over the entire range of concentrations tested. A summary of the of validation and uncertainty data for each compound were given in Table 3.

Moreover, the intra-assay precision was evaluated by performing 9 replicates per day while the inter-assay precision was evaluated analyzing fresh extract samples each day in 3 consecutive days. Table 4 shows the intra and inter-assay precision using three different concentrations of the analyte. The precision for the intra-assay was more that 88% while for the inter-assay was more than 98%.

LC-MS analysis of crude extracts

We assess the polyphenols profile of three different crude extracts *Clematis flammula* L. leaves (CFL), *Pistacia lentiscus L.* black fruits (PBF) and *Populus nigra* L. (PN). According to Table 5, 15 polyphenols were detected in the extract CFL, while 11 polyphenols were detected in PBF and 9 polyphenols were detected in the PN extract. This difference potentially is related to polyphenols variation in different plant extracts.

Table 4
Precision results based on LC-ESI-MS method.

Analyte	Target value (ug/L)	Intra-assay		Inter-assay	
		Precision	RSD%	Precision	RSD%
Chrysin	0.05	89.1	4.9	100.1	8.5
	1	98.1	2.1	100.01	9.2
	10	99.1	1.4	100.1	9.1
Pyrogallic Acid	0.05	90.1	4.2	100.1	8
	1	95.1	1.9.	99..01	4.1
	10	90.1	1.2	100.0	2.1
Catechol	0.05	88.9	4.9	99.8	4.5
	1	92.1	2.1	99.2	2.3
	10	93.1	1.2	99.2	4.2
C.Tannic Acid	0.05	90.1	5.1	100.0	6.1
	1	92.1	3.1	101.01	2.1
	10	94.1	1.5	100.1	4.1
Rutin	0.05	91.1	4.8	99.2	8.5
	1	94.1	2.0	99.4	9.1
	10	96.1	1.2	100.4	4.2
Citric acid	0.05	90.1	5.9	98.9	8.7
	1	93.1	3.1	101.1	5.6
	10	95.1	2.4	101.2	8.2
Curcumin	0.05	99.1	1.9	100.1	7.5
	1	98.9	2.1	98.9	4.2
	10	94.2	1.2	99.8	6.7
Tannic Acid	0.05	88.1	4.9	99.9	8.2
	1	89.1	5.1	98.8	9.1
	10	94.1	3.4	100.0	4.5
Catechin	0.05	92.1	7.9	101.1	6.4
	1	94.1	3.1	99.01	9.1
	10	95.1	3.4	100.1	4.3
Silymarin	0.05	92.1	4.9	98.9	5.8
	1	95.1	5.1	99.9	8.9
	10	97.1	2.4	100.1	8.4
Diosmin	0.05	93.1	2.9	98.9	8.2
	1	95.1	7.1	101.01	4.2
	10	97.1	2.4	100.2	5.6
Kaempferol	0.05	94.1	2.9	101.2	5.8
	1	97.1	5.1	100.2	7.2
	10	99.1	2.4	99.9	7.1
Coumaric Acid	0.05	93.1	4.9	100.1	4.6
	1	96.1	4.1	101.01	6.7
	10	95.1	3.4	100.1	3.5
Ascorbic Acid	0.05	89.4	6.9	99.9	4.2
	1	97.9	4.1	98.9	7.1
	10	98.1	3.4	101.1	4.2
cyanidin	0.05	92.3	7.9	100.1	6.5
	1	93.1	5.1	99.9	7.1
	10	98.4	4.4	100.1	8.4
Cinnamic acid	0.05	99.1	5.9	101.1	8.2
	1	95.6	3.1	99.9	9.1
	10	97.2	2.4	101.1	4.7
Gallic Acid	0.05	89.4	6.9	100.1	8.1
	1	94.1	3.1	100.9	10.1
	10	97.1	2.4	100.1	5.8
Caffeic Acid	0.05	89.7	6.9	99.9	4.3
	1	97.9	3.1	101.2	5.1
	10	99.3	5.4	101.2	6.9

Additional information

Polyphenols are secondary metabolites widely known for their antioxidant properties that are found in a wide variety of foods or plants [4,6]. Polyphenols are divided in different groups of compounds according to their chemical structure [7]. Numerous of studies have underlined the important role of polyphenols in human health and particularly, in diseases related to oxidative stress [8,9]. In addition, several studies emphasize the significant role of polyphenols in the delay of the development of cancer, cardiovascular and neurodegenerative diseases [10]. Besides that, there is a growing interest in the use of polyphenols in food or cosmetic industries [11,12] as the market demand of using natural ingredients is constantly growing. Thus, the use of polyphenols-based extracts as natural additives is an important strategy for the industrial manufacturers [13,14].

Table 5
Detection and Quantification of polyphenols in different plant extracts.

Mixture of Stds	*Clematis flammula L* extract (ug/L)	*Pistacia lentiscus L.* extract (ug/L)	*Populus nigra* L. extract (ug/L)
Chrysin	n.d.	10 ± 0.12	5 ± 0.01
Pyrogallic Acid	1	1 ± 0.11	1 ± 0.04
Catechol	10 ± 0.10	n.d.	n.d.
Conjugated Tannin Acid	1	1 ± 0.01	1 ± 0.09
Rutin	10 ± 0.01	1 ± 0.10	1 ± 0.02
Citric acid	5	10 ± 0.12	n.d.
Curcumin	50	10 ± 0.11	n.d.
Tannic Acid	n.d.	n.d.	1 ± 0.02
Catechin	50 ± 0.21	n.d.	n.d.
Silymarin	50 ± 0.15	n.d.	25 ± 0.11
Diosmin	50 ± 0.11	50 ± 0.21	n.d.
Kaempferol	1 ± 0.10	1 ± 0.01	1 ± 0.01
Coumaric Acid	n.d.	1 ± 0.01	1 ± 0.01
Ascorbic Acid	1 ± 0.02	n.d.	n.d.
cyanidin	50 ± 0.21	25 ± 0.21	n.d.
Cinnamic acid	3 ± 0.02	n.d.	n.d.
Gallic Acid	3 ± 0.01	3 ± 0.01	3 ± 0.01
Caffeic Acid	3 ± 0.01	n.d.	n.d.

The extraction method as well as the characterization of polyphenols is quite complicated as these compounds can be found in plain or highly complex structures, such as the plant-based matrixes. In this work, deep eutectic solvents (DES) have been applied as extraction solvents for polyphenols. DES are considered green solvents with a growing interest to the scientific community. DES have high biodegradability, low toxicity and ease of handling. Nevertheless, the use of DES for polyphenols recovery is challenging as they can establish a strong hydrogen bond network [15]. Alternatively, another possibility is to use DES as solvent extraction as well as formulation medium. In this way, DES can be suitable for cosmetics, pharmaceutical, or food applications [15].

In addition, LC-MS or LC-MS/MS approaches provide a metabolomic profile of plant extracts requiring the minimum of sample preparation and a high-resolution determination of metabolites [16–18]. With this in mind, the main objective of this study was to develop a simple method based on LC-MS, as LC-MS/MS methods are not always available in laboratories, all so to assess nutraceutical or pharmaceutical potential of these plant extracts at a later stage. Moreover, these neoteric solvents, DES, we used for the preparation of the extracts would certainly help to alleviate some of the current technical issues. Furthermore, for industrial purposes it is quite important to consider some of the properties such as viscosity or the very low vapor pressure that preclude industrial transfer.

In our study, we developed a simple analytical method based on the LC-ESI/MS method so as to determine and quantify 18 different polyphenols such as phenolic acids, anthocyanins and flavonoids. For the majority of the compounds the recovery was higher than 80% while the LODs ranged between 0.4–1ug/L. Moreover, we were able to detect difference polyphenols among the different plant extracts and as such the *Clematis flammula* L. leaves (CFL) extract was richer in polyphenols compared to the *Pistacia lentiscus* L. black fruits (PBF) and *Populus nigra* L. (PN) extracts.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Sophia Letsiou: Conceptualization, Methodology, Writing – review & editing. **Maria Trapali:** Conceptualization, Methodology, Writing – review & editing. **Sara Oumenoune Tebbi:** Writing – review & editing. **Nadjet Benaida-Debbache:** Writing – review & editing.

Data availability

No data was used for the research described in the article.

Ethics statements

Our work does not involve human subjects, animal experiments and data collected from social media platforms.

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