Research Article
doi.org/10.1002/ansa.202400006
Chemistry Europe
European Chemical Societies Publishing

Received: 15 February 2024
Revised: 26 July 2024
Accepted: 3 August 2024

Application of a kosmotrope (Na_2CO_3) and chaotrope (NaCl) in chemometric optimization of aqueous two-phase extraction of bioactive compounds in *Hypoxis iridifolia*

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Funding information
South African Agency for Science and Technology Advancement

Abstract

Enterolactone, coumaric acid and vitexin are polyphenolic compounds present in a variety of fruits, vegetables, cereals and plants. These bioactive compounds are in high demand due to their antioxidant property in various tissues and organs. The purpose of this study was to develop a simultaneous extraction method, an aqueous two-phase extraction (ATPE) method, that would enable the extraction of these compounds from *Hypoxis iridifolia*. This environmentally friendly extraction method only applied water and ethanol as extraction solvents for these analytes from the plant matrix. After phase separation, the analytes were salted-out from the aqueous phase into the organic phase with the aid of a chaotrope (NaCl) or kosmotrope (Na_2CO_3). Thereafter, the analytes were withdrawn by a micro-pipette for analysis on the high-performance liquid chromatography–photodiode array detector. Optimization was conducted using a central composite design, where three parameters were examined which involved percentage ethanol, centrifugation time and salt type. Generally, the optimized conditions for extraction were an ethanol percentage of 100% and a centrifugation time of 10 min, which yielded concentrations of 2942, 23,823 and 8881 mg kg^{-1} for enterolactone, vitexin and coumaric acid, respectively, in the presence of a kosmotrope. The optimized conditions of extraction in the presence of chaotrope were an ethanol percentage of 66% and a centrifugation time of 10 min with concentrations of 6727, 20,833 and 8618 mg kg^{-1} for enterolactone, vitexin and coumaric acid, respectively. The ATPE method involving Na_2CO_3 was a better extractant of all the compounds studied relative to that of NaCl. The superior extraction capability of Na_2CO_3 in ATPE could serve as a prototype for the development of efficient extraction methods to meet the high demand for medicinal compounds derived from natural products.

List of Abbreviations: ATPE, aqueous two phase extraction; CCD-RSM, central composite design–response surface model; H. iridifolia, Hypoxis iridifolia; HPLC-PAD, high-performance liquid chromatography–photodiode array detector; QuEChERS, Quick, Easy, Cheap, Effective, Rugged and Safe; UV–Vis, ultra violet–visible.

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KEYWORDS
aqueous two-phase extraction, coumaric acid, enterolactone, *Hypoxis iridifolia*, vitexin

1 | INTRODUCTION

Plants are a natural hub for structurally diverse secondary metabolites containing unique biological functions and potencies as drug candidates. As a result, researchers have continuously sought to improve the extraction of metabolites from natural products. One of the commonly applied extraction methods is the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) technique reported by Anastassiades et al.[1] for the extraction of pesticides from food matrices. The same technique was later validated by Lehotay et al. for the extraction of 229 pesticides from fruits and vegetables.[2] Despite being a popular extraction method, the QuEChERS technique also has its setbacks. The method has often shown reliance on toxic organic solvents such as acetonitrile and requires multiple steps for sample preparation making it tedious and costly.[3] Additionally, other commonly used methods such as supercritical fluid extraction and microwave-assisted extraction often suffer from the requirement for elevated pressure and high equipment costs.[4,5]

Owing to the potential medicinal applications of naturally derived metabolites, there is a need to preconcentrate these compounds using robust methods of extraction to improve extraction time while simultaneously achieving good recoveries. Extraction of phytoconstituents from plants is dependent on a variety of factors, among which include the use of chaotropic and kosmotropic salts. Chaotropes are salts that disrupt hydrophobic interactions of plant-derived compounds in water, hence allowing for dissolution of non-polar compounds in water. They are also weakly hydrated compounds and generally consist of large singly charged ions. On the contrary, kosmotropes do not interfere with hydrophobic interactions and are strongly hydrated as a result of their structural design, which consists of small multiply charged ions.

Aqueous two-phase extraction (ATPE) is a liquid–liquid partitioning method where one layer may be composed of a salt-saturated aqueous layer and the other an organic layer or as in other ATPE systems; a salt–salt or an ionic liquid–salt system.[6] Some of the main uses of ATPE systems include separation, purification and enrichment of metabolites.[6] The major advantages of ATPE are high capacity, biocompatible environment, low interfacial tension of phases, high yields and low process time.[6–7]· Additionally, this technique uses salts that allow for partitioning of ethanol (green solvent) from water, where the ethanol layer is enriched with metabolites. Hence, the aim of this work was to determine if ATPE using the salting-out technique, in the presence of NaCl (chaotrope) or Na_2CO_3 (kosmotrope) would enable efficient extraction of enterolactone, coumaric acid and vitexin (see Figure 1) from leaves of *Hypoxis iridifolia*, a medicinal plant reported for anti-tuberculosis activity.[8] By coupling ATPE with high-performance liquid chromatography–photodiode array detector (HPLC-PAD) analysis, a rapid and sensitive method for the determination of *H. iridifolia* nutraceutical compounds was established.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

The salts NaCl (anhydrous ≥99% purity), Na_2CO_3 (anhydrous ≥99% purity) and ethanol (99% CP) were purchased from Associated Chemical Enterprises (Johannesburg, South Africa) and Sigma-Aldrich (Johannesburg, South Africa). Ultra-pure water (0.005 µS, 18 mΩ) using a direct-Q 5UV distiller (Millipore, Danvers, MA, USA) was applied for the preparation of the salt solutions. *H. iradifolia* was purchased at Random Harvest Nursery (Johannesburg, South Africa). Individual standard solutions of enterolactone (5000 µg mL^{-1}, in methanol), vitexin (5000 µg mL^{-1}, in methanol) and coumaric acid (5000 µg mL^{-1}, in methanol) were purchased from Sigma-Aldrich (Modderfontein, South Africa). Liquid chromatography-mass spectrometry grade water and methanol were purchased from Sigma-Aldrich (Modderfontein, South Africa).

2.2 | ATPE procedure

The ATPE procedure is summarized in the following steps. Ground leaves of *H. iridifolia* were stored in glass containers and covered in paper bags to prevent light penetration. Thereafter, ground leaves of *H. iridifolia* (500 mg) were weighed and placed in centrifuge tubes (50 mL) containing ethanol with concentrations ranging from 0% to 100%. The mixture was then shaken on a DIAB MX-RL-Pro dragon shaker at 70 rpm for 12 h at 25°C for the extraction of the metabolites. The mixtures were then centrifuged for 10–60 min to enable phase separation. Following the formation of two layers, the upper layer which contained the analytes of interest was withdrawn and injected into a vial followed by analysis on the HPLC-PAD.

2.3 | Calibration curves

The calibration curves for enterolactone, vitexin and coumaric acid were determined. For enterolactone, the R^2 = 0.9906, vitexin the R^2 = 0.8672 and for coumaric acid the R^2 = 0.8752. The closer the R^2 value is to 1, the more reliable the linear calibration curves for the quantification of those respective compounds. Further information on the elution and absorption profile of standards used is included in the Supporting Information (Table S1).

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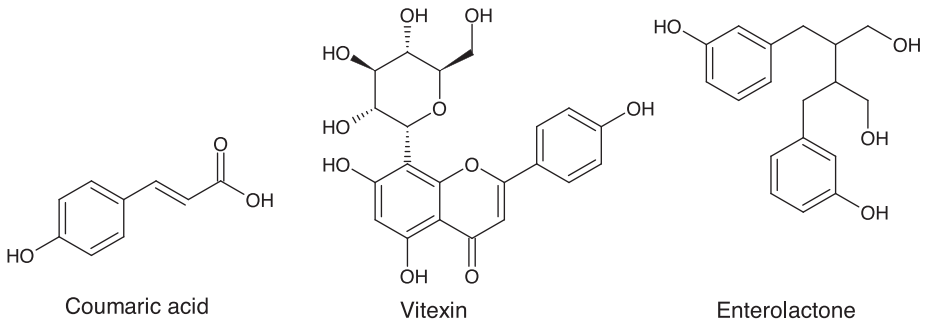

FIGURE 1 Chemical structures of the bioactive compounds studied.

2.4 | Statistical analysis

The central composite design–response surface model (CCD-RSM) was fitted to experimental data to obtain the relationship between factors and optimize the response of *Z* (enterolactone, vitexin and coumaric acid) in relation to *A* (percentage ethanol) and *B* (centrifugation time) using Design Expert 11 (Stat-Ease, Minneapolis, MN, USA). By using CCD, a total of 36 experimental runs, done in triplicate (Table 1), were designed which included three numerical factor levels for percentage ethanol (0, 50 and 100%), three-factor numerical levels for centrifugation time (10, 35 and 60 min) and two categorical factor levels for salts which included the chaotrope (NaCl) and kosmotrope (Na_2CO_3).

The interaction between the various parameters studied, and its resultant effect on the extraction of enterolactone, vitexin and coumaric acid (mg kg^{-1}) was fitted to experimental data by using a statistical multiple regression approach method of least square and resulted in the lowest possible residual.[9] Model parameters and model significance were determined at $p < 0.05$. The fitness of the model was determined by evaluating the coefficient of regression (R^2) obtained from the analysis of variance. The model fit generated the response surface that defined the behaviour of the response variable. Using these plots, the optimized ranges for each factor lead to the highest response (i.e., concentration of enterolactone, vitexin and coumaric acid) that can be extracted.[9,10]

3 | RESULTS AND DISCUSSION

3.1 | Identification of enterolactone, vitexin and coumaric acid based on retention times and adsorption wavelengths

In this study, the extraction of enterolactone, vitexin and coumaric acid was performed, which was reported to be contained in *H. iridifolia*, using ATPE.[11,12] The ATPE approach was improved by applying various parameters as shown in Table 1, on the recovery of the target metabolites. Various extraction methods and characterization methods have been applied for the enrichment of enterolactone, vitexin and coumaric acid from natural products. For instance, vitexin was extracted from freeze-dried powdered flax of (*Linum usitatissimum L.*) using methanol/water (5:1, v/v) and separated on a C18 column with a retention time and λ_{max} of around 10 min 20 s and 278 nm, respectively.[13] A study by Glavnik et al. evaluated the high-performance liquid chromatography performance of metabolites in bacterial cultures.[14] In their investigation, UV–Vis studies revealed that *p*-coumaric acid had an λ_{max} at 566 nm.[14] The λ_{max} of enterolactone during HPLC quantification was determined to be 283 nm in a study conducted by Ayella et al.[15]

From the concentrations of enterolactone, vitexin and coumaric acid obtained in Table 1, the ATPE technique was more favourable for the extraction of vitexin. Since extraction was carried out from grounded leaves of *H. iradifolia*, this may indicate that vitexin is prevalent in leaves of *H. iradifolia*. However, this should not discount that there may be other factors at play which include amongst others the higher affinity of vitexin for ethanol. This bioactive compound has a variety of pharmacological effects which include antioxidant, anti-inflammatory and anti-bacterial effects.[16,17] Vitexin has been reported to be present in mung bean (*Vigna radiate*),[18–19] common buckwheat (*Fagopyrum esculentum*) and hawthorn (Rosaceae).[20–21] Not much is known about the presence or extraction of vitexin from plants within the Hypoxidaceae family. Hence, based on the data in Table 1, the leaves of *H. iradifolia* were studied as a viable source of vitexin.

Figure 2A shows a chromatogram of the sample containing the crude extract with peaks denoted as 1, 2, 3 and 4 at a wavelength of 190 nm. Figure 2B–D shows the crude extract exposed to wavelengths of 254, 284 and 584 nm, respectively. Figure 2E–2G presents chromatograms of analytical standards of enterolactone (1000 µg L^{-1}), vitexin (1000 µg L^{-1}) and coumaric acid (1000 µg L^{-1}) analysed at λ_{max} of 254, 284 and 584 nm, respectively. Furthermore, Figure 2E–G shows peaks at retention times of 8.2, 9.1 and 9.8 min corresponding to peaks 2, 3 and 4 in the crude extract (Figure 2A). Hence, peaks 2, 3 and 4 indicated the target compounds enterolactone, vitexin and coumaric acid. The methanol solvent front is annotated as 1.

3.2 | Fit statistics of experimental and predicted data

The model fitted to the data was observed to have a quadratic fit; *p* values less than 0.05 ($p = 0.008$), indicating that the model terms

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TABLE 1 Central composite design of experiments for ATPE of bioactive compounds.

Number	Percentage ethanol	Centrifugation time (min)	Partitioning salts	Enterolactone (mg kg^{-1})	Vitexin (mg kg^{-1})	Coumaric acid (mg kg^{-1})
1	50	35	NaCl	2456 ± 15	17,900 ± 52	4424 ± 21
2	50	35	Na_2CO_3	1283 ± 13	16,505 ± 49	6516 ± 20
3	50	35	Na_2CO_3	1343 ± 14	16,533 ± 51	7501 ± 19
4	100	60	NaCl	4332 ± 17	18,433 ± 55	6544 ± 23
5	50	35	NaCl	2410 ± 19	17,647 ± 54	7732 ± 22
6	50	35	NaCl	2788 ± 20	17,593 ± 53	7685 ± 25
7	0	35	Na_2CO_3	–	–	–
8	100	10	NaCl	3911 ± 23	21,448 ± 56	2704 ± 27
9	0	10	NaCl	2087 ± 24	18,449 ± 48	7824 ± 19
10	50	35	NaCl	2878 ± 23	18,239 ± 50	8650 ± 18
11	100	60	Na_2CO_3	–	23,872 ± 49	2094 ± 24
12	0	10	Na_2CO_3	2372 ± 18	17,694 ± 51	7566 ± 26
13	50	35	Na_2CO_3	3087 ± 21	20,667 ± 49	10070 ± 23
14	50	35	NaCl	2950 ± 19	22,516 ± 50	9220 ± 30
15	50	35	NaCl	3064 ± 25	21,731 ± 47	8598 ± 28
17	100	35	NaCl	–	–	–
18	50	35	NaCl	3725 ± 15	18,834 ± 55	–
19	50	60	NaCl	2422 ± 24	20,035 ± 58	7384 ± 29
20	0	35	NaCl	1541 ± 15	20,700 ± 53	9926 ± 30
21	50	35	Na_2CO_3	1101 ± 18	19,912 ± 57	7730 ± 31
22	100	10	Na_2CO_3	3622 ± 27	21,369 ± 58	12140 ± 35
23	50	10	NaCl	3435 ± 22	19,666 ± 57	7185 ± 28
24	50	35	Na_2CO_3	2776 ± 25	19,385 ± 56	7395 ± 30
25	50	35	NaCl	2847 ± 30	18,995 ± 51	7757 ± 31
26	50	10	Na_2CO_3	2894 ± 31	20,683 ± 47	7552 ± 27
27	50	35	Na_2CO_3	2939 ± 16	20,679 ± 54	8306 ± 30
28	50	60	Na_2CO_3	2159 ± 18	20,755 ± 52	8427 ± 35
29	50	35	NaCl	2816 ± 21	19,915 ± 53	8142 ± 36
30	50	35	NaCl	2936 ± 20	20,526 ± 48	8622 ± 29
31	50	35	Na_2CO_3	2763 ± 24	21,555 ± 50	8267 ± 31
32	0	60	NaCl	2482 ± 25	19,934 ± 51	7026 ± 25
33	0	60	Na_2CO_3	2291 ± 17	17,222 ± 52	7305 ± 28
34	50	35	Na_2CO_3	2838 ± 24	21,536 ± 44	7785 ± 29
35	50	35	Na_2CO_3	2574 ± 25	21,514 ± 48	8485 ± 32
36	50	35	Na_2CO_3	2643 ± 22	21,951 ± 55	8003 ± 35

are significant. Furthermore, the goodness of fit between the experimental and the predicted values was $R^2 = 0.7$. The Pareto charts examining the effects of the parameters *A* is percentage ethanol and *B* is centrifugation time on the extraction of (a) enterolactone, (b) vitexin and (c) coumaric acid are included in Figure 3A–C. The quadratic effect of percentage ethanol (A^2) was found to have a significant effect ($p < 0.05$) on the extraction of enterolactone ($p = 0.007$), vitexin ($p = 0.003$) and coumaric acid ($p = 0.028$) (Figure 1). This also indicates that the quadratic effect percentage ethanol was influential (significant) in the following order: coumaric acid < vitexin < enterolactone. Another efficient parameter was the quadratic effect of centrifugation time (B^2) for enterolactone ($p = 0.017$) and vitexin ($p = 0.010$). The linear effect of percentage ethanol (*A*) and centrifugation time (*B*) was found to be insignificant ($p > 0.05$). This indicated that the quadratic parameters had an effect on the extraction of the analytes while the linear parameters had minimal or no effect on

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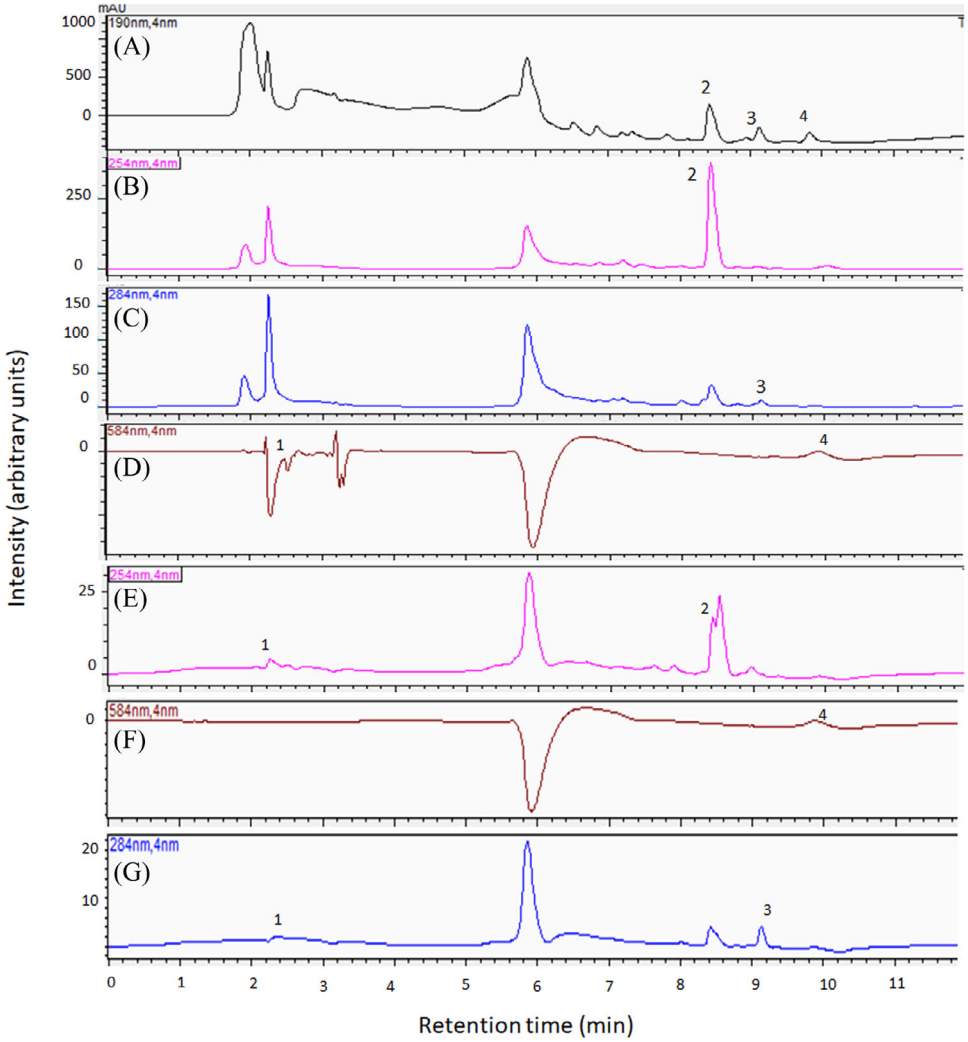

FIGURE 2 The chromatogram of the crude extract containing the eluted compounds at a wavelength of 190 nm (A), 254 nm (B), 284 nm (C) and 584 (D). The chromatograms of the analytical standards enterolactone, vitexin and coumaric acid are shown in E, F and G, respectively.

extraction, a similar observation was reported by Mokgehle et al.[12] and Gbashi et al.[22]

3.3 | Response surface equations and corresponding to NaCl and Na_2CO_3 and the resultant optima

Response Equations (1) and (2), corresponding to NaCl and Na_2CO_3, respectively, and the resultant response surfaces evaluating the multivariate interaction between percentage ethanol and centrifugation time are shown in Figure 4. Equations (1) and (2) are the response surface equations for NaCl and Na_2CO_3, respectively, where A is percentage ethanol; B is centrifugation time and Z is extraction yield (mg kg^{-1}).

NaCl

$$Z = 18523 + 13.01A + 41.1B - 0.44AB - 0.05A^2 - 0.16B^2, \quad (1)$$

Na_2CO_3

$$Z = 18455 + 61.76A - 87.31B + 0.21AB - 0.08A^2 + 1.11B^2. \quad (2)$$

The optimum extraction conditions for vitexin in the presence of NaCl (Figure 4A) were observed at a centrifugation time of approximately 60 min and percentage ethanol of 1% with a yield of approximately 20,000 mg kg^{-1} (Table 2). For Figure 4B, in the presence of Na_2CO_3, as both centrifugation time and percentage of ethanol were increased, the optimal concentration of vitexin obtained was approximately 24,000 mg kg^{-1} (Table 2). A comparison of Figure 4A and 4B indicated that Na_2CO_3 was a better extractor of vitexin than NaCl. This is because the doubly charged carbonate ions from Na_2CO_3, probably formed stronger hydrogen bonds with the solvation sphere surrounding vitexin than singly charged chloride ions, enhancing the extent of its precipitation (salting-out) from the hydration sphere and its subsequent extraction by ethanol. The superior charge density of the carbonate ion in relation to the chloride ion facilitated the extraction of

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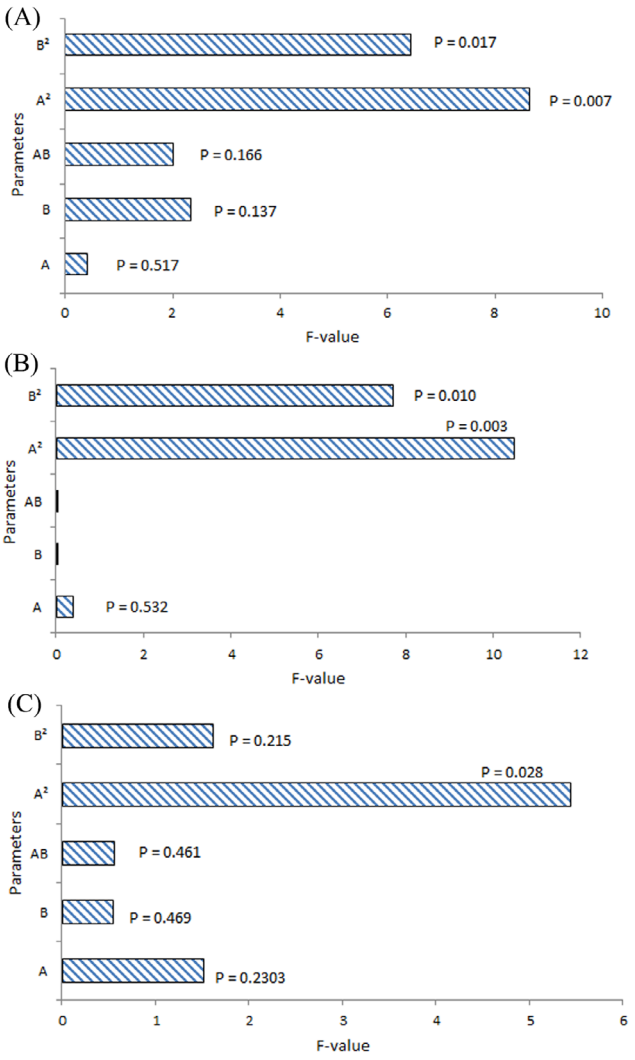

FIGURE 3 Pareto charts of (A) enterolactone, (B) vitexin and (C) coumaric acid.

vitexin when Na_2CO_3 was applied.[12] This observation is in agreement with the Hofmeister series as discussed by Kang et al.,[23] Dogra et al.,[24] and Wang et al.[25]

The extraction of coumaric acid was evaluated in the presence of (a) NaCl and (b) Na_2CO_3. In Figure 5A, the optimal extraction of coumaric acid is achieved at a centrifugation time of approximately 35 min and percentage ethanol of 30%. For Figure 5B, in the presence of Na_2CO_3, when both centrifugation time and percentage of ethanol were increased, an optimum concentration of approximately 9000 mg kg^{-1} (Table 2) was obtained. The application of multiply charged salts such as the kosmotrope (Na_2CO_3) was shown to be a comparably better extractant of coumaric acid than the chaotrope (NaCl). This observation is due to the Hofmeister series as discussed in Figure 4A,B and is similar to what Bulgariu and Bulgariu,[26] Neves et al.,[27] and Hyde et al.[28] reported on the better salting-out capacity of SO_4^{2-} compared to Cl^-.[27]

The response surface equations corresponding to NaCl and Na_2CO_3 are shown in Equations (3) and (4), and the resultant response surfaces evaluating the multivariate interaction between percentage ethanol and centrifugation time are shown in Figure 5A and 5B, respectively. Equations (3) and (4) are the response surface equations for NaCl and Na_2CO_3 respectively, where *A* is percentage ethanol; *B* is centrifugation time and *Z* is extraction yield (mg kg^{-1}).

TABLE 2 Optima obtained for the analytes during ATPE.

Analytes	Centrifugation time (min)	Percentage ethanol (%)	Predicted yield (mg kg^{-1})
Optimal extraction conditions in NaCl			
Vitexin	60	1	20,833
Coumaric acid	32	1	8,618
Enterolactone	60	10	6,727
Optimal extraction conditions in Na_2CO_3			
Vitexin	60	100	23,823
Coumaric acid	60	60	8,881
Enterolactone	10	35	2,942

NaCl

$$Z = 8079.23 - 49.82A + 47.95B + 0.39AB + 0.14A^2 - 0.91B^2, \quad (3)$$

Na_2CO_3

$$Z = 7602 + 69.72A - 3.39B + 0.45AB - 1.49A^2 - 0.02B^2. \quad (4)$$

NaCl (Figure 6A) and Na_2CO_3 (Figure 6B) were evaluated for the extraction of enterolactone. It was noted that as centrifugation time was decreased, and percentage ethanol was increased, and the optimum concentration of 6700 mg kg^{-1} was obtained (Figure 6A) (Table 2). In Figure 6B as centrifugation time is decreased and percentage of ethanol is increased, the optimum concentration of 4000 mg kg^{-1} was obtained. In contrast to the observations for vitexin and coumaric acid in Figures 4A,B and 5A,B, respectively, NaCl was a better extracting agent than Na_2CO_3. This could possibly be that salting out induced by the superior charge capability of Na_2CO_3 was limited. An additional factor that could have influenced the inferior performance of Na_2CO_3 for the extraction of enterolactone is the dilute solvent (<50% ethanol). At ethanol concentrations of less than 50%, there is a negative contribution to the extraction of enterolactone due to salt-mediated hydrolysis, which resulted in the degradation of enterolactone.[29]

The response surface equations corresponding to NaCl and Na_2CO_3 are shown in Equations (5) and (6), and the resultant response surfaces evaluating the multivariate interaction between percentage ethanol and centrifugation time are shown in Figure 6A,B. Equations (5) and (6) are the response surface equations for NaCl and Na_2CO_3 respectively, where *A* is percentage ethanol, *B* is centrifugation time, and *Z* is extraction yield (mg kg^{-1}).

NaCl

$$Z = 2073.24 + 24.59A - 19.21B - 0.56AB + 0.29A^2 + 0.38B^2, \quad (5)$$

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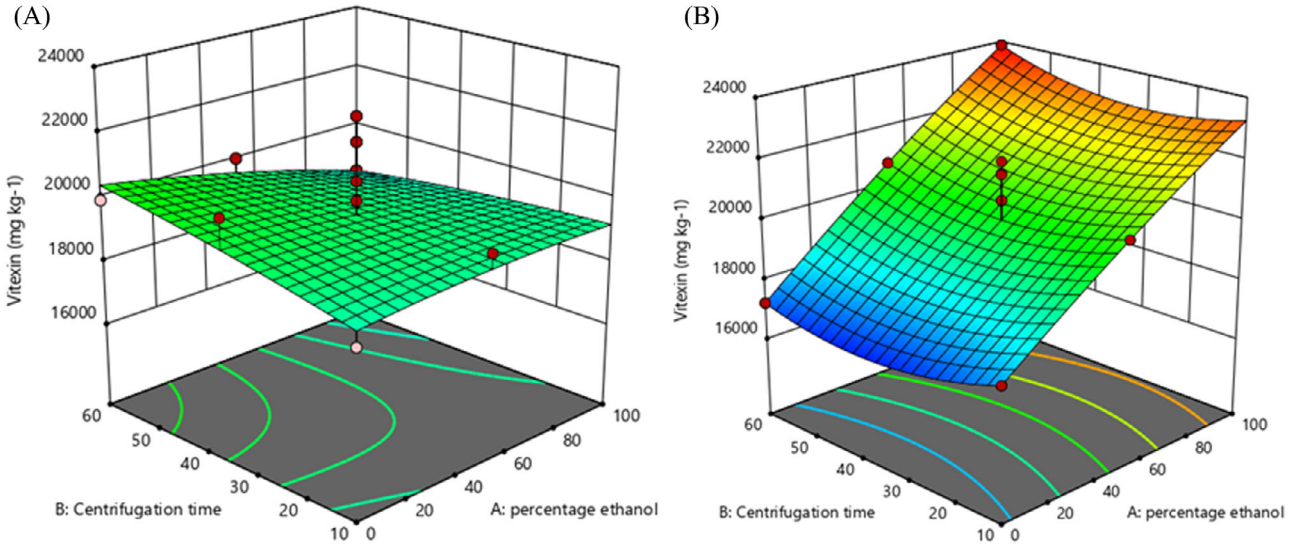

FIGURE 4 RSM for vitexin using (A) NaCl and (B) Na_2CO_3.

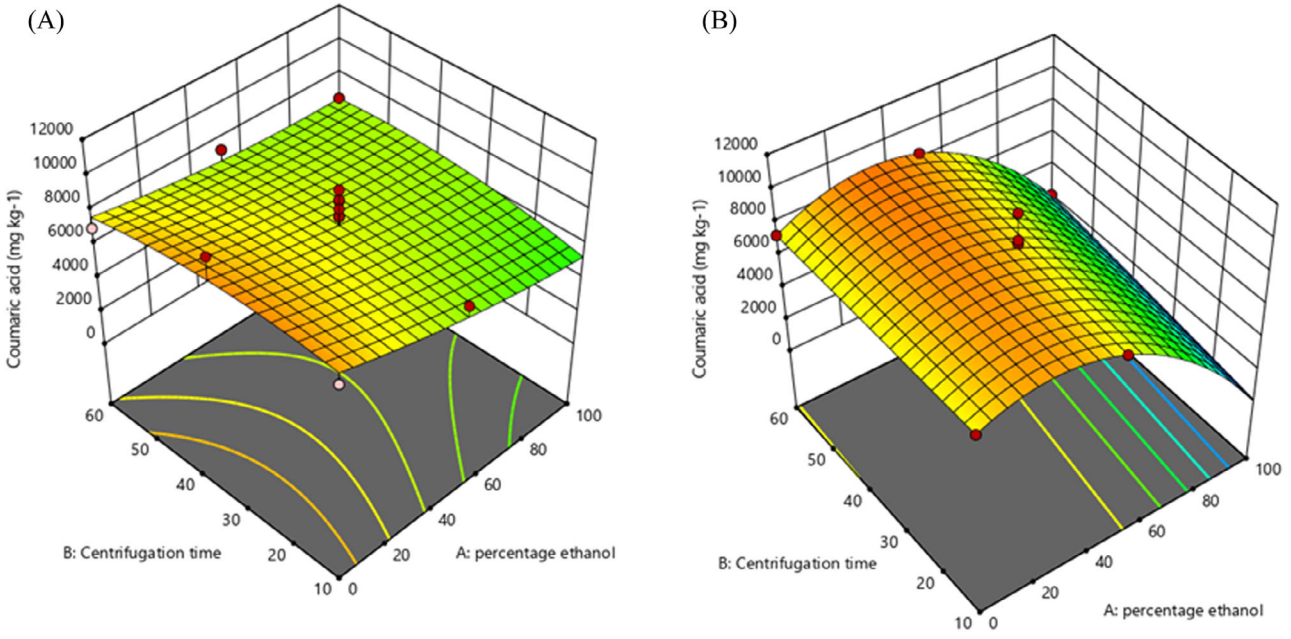

FIGURE 5 RSM for coumaric acid using (A) NaCl and (B) Na_2CO_3.

Na_2CO_3

$$Z = 2572 + 33.32A - 23.01B - 0.26AB - 0.41A^2 + 0.306B. \quad (6)$$

4 | CONCLUSIONS

The ATPE technique based on the salting-out method was shown to be an efficient approach for the simultaneous extraction of multiple metabolites from *H. iridifolia*. The ATPE method was more efficient for the extraction of vitexin and coumaric acid when the kosmotrope (Na_2CO_3) was used, with optimal concentrations of 23,823 and 8881 mg kg^{-1}, respectively. The quadratic effect of percentage ethanol (A^2) was an influential (significant) parameter in the extraction of all the analytes studied. The greater charge density of the carbonate ion relative to the chloride ion, generally enabled for the higher extraction of the studied analytes when Na_2CO_3 was used as a salting-out agent compared to NaCl. The application of a green solvent (ethanol) and water in the ATPE method offered an environmentally friendly alternative to the toxic solvents traditionally used in extraction. The ATPE method, under the conditions studied, was useful for the extraction of bioactive compounds. This has positive implications in the pharmaceu-

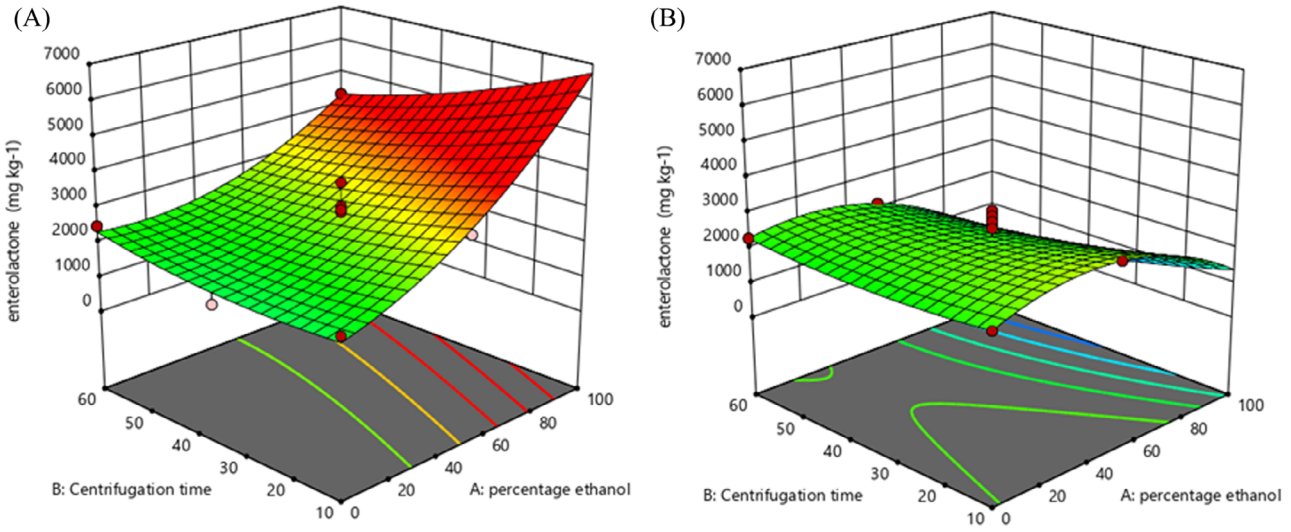

FIGURE 6 RSM for enterolactone using (A) NaCl and (B) Na_2CO_3.

tical fraternity as similar user-friendly methods can be developed to better extract other bioactive compounds from natural products.

ACKNOWLEDGEMENTS

Special thanks to the National Research Foundation for funding.

CONFLICT OF INTEREST STATEMENT

Tebogo Mphatlalala Mokgehle declares that he has no competing interests. Rangani Tracy Lukheli declares that she has no competing interests. Nikita Tawanda Tavengwa declares that he has no competing interests.

DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this published article.

FUNDING INFORMATION

University of Venda, Sasol Inzalo and the National Research Foundation

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SUPPORTING INFORMATION

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How to cite this article: Lukheli RT, Tavengwa NT, Mokgehle TM. Application of a kosmotrope (Na_2CO_3) and chaotrope (NaCl) in chemometric optimization of aqueous two-phase extraction of bioactive compounds in *Hypoxis iridifolia*. *Anal Sci Adv*. 2024;5:2400006.
https://doi.org/10.1002/ansa.202400006