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abstract

Often, osmotic shock, oxidative stress and de-compartmentalization in dried plant cells negatively impact efficacy, bioavailability, functional properties, safety and reproducibility of conventional botanical extracts. This prompted the development of a sustainable solvent-free Zeta Fraction Technology (ZFT), which is based on scientific principles discovered by Van't-Hoff and Debye, and on Derjaguin-Laundau-Verwey-Overbeek (DLVO) theory. Progress in life sciences and in instrumentation for broadband dielectric spectrometry has contributed to the development of ZFT. This technology includes: collection of living plants with maximum metabolic activity; separation of relatively stable intracellular colloidal dispersion (ICD) from cell walls; treatment of ICD to engage particular components of the dispersed phase and continuous phase in specific interactions by changing the balance between repulsive and attractive forces; and separation of ICD to different Zeta Fractions. Plant examples including Sacred Lotus (*Nelumbo nucifera*), Tea Plant (*Camellia sinensis*), Feverfew (*Chrysanthemum parthenium*) and Sage (*Salvia officinalis*) demonstrate that ZFT allows targeting multiple pathways with a single ingredient, improving safety by removing undesirable components and chemicals of concern, all while resulting in minimal environmental impact and waste. ZFT utilizes the underexplored potential of living plants and may be used in combination with existing extraction technologies to achieve effective volume reduction.

Introduction

Most conventional botanical extraction processes begin when plants are harvested and dried. The reproducibility of dried plant material composition is impacted by variability in growth, harvest, and drying conditions. Even relatively low-variability consecutive single factors could, when combined, significantly contribute to low reproducibility of composition and properties of dry material. Thus given a favorable 80 % rate of reproducibility at each of the three major steps (growth, harvest and drying), dry plant raw material variability of about 50 % (0.8 × 0.8 × 0.8 = ~0.5) could occur [1]. In addition, the drying process initiates unwanted osmotic shock and oxidative stress, which can trigger de-compartmentalization in plant cells, disruption of enzymes, hydrolysis, polymerization of phenols, transformation of glycosides to aglycones, generation of products of the Maillard reaction, isomerization and/or microbial contamination. As a result, only catabolites and relatively stable compounds in dried plants that could be called "survivors of catabolic transformations" are accessible for conventional extraction. These conditions negatively impact the reproducibility, efficacy, bioavailability, and safety profile of many finished extracts. Additionally, the resulting compositions of extractives are limited by their affinity to particular solvents. Thus, a conventional extraction approach does not allow for the capture of the whole spectrum of active materials existing in living plant cells; and also it could destroy natural synergistic interactions of different types of compounds. As a result, desirable multifunctional properties of natural compounds can be diminished or lost. For example, a synergistic complex of berberine and 5'-methoxyhydnocarpin discovered in living barberry plants could not be accessed via a conventional extraction approach [2]. Importantly, conventional botanical extraction often uses ion-exchange resins and high volumes of organic solvents, (sometimes tens of liters per 1 kg of extract [3]), with significant energy consumption which is further increased if solvent evaporation and regeneration are utilized. It should be noted that supercritical CO_2 extraction is merely the replacement of hexane extraction, not a wide-ranging solution. This prompted the development of sustainable solvent-free Zeta Fraction Technology (ZFT) [4-8].

Zeta Fraction Technology

Living plants having maximum metabolic activity represent the best source for capturing the whole spectrum of natural complexes and compounds. Internal homeostasis in living cells maintains major physico-chemical characteristics of plant material within relatively narrow ranges – better than any drying and storage methods. Because the viability of plants is determined by their photosynthetic activity, verifying it (and thus many other parameters) is an important quality control factor utilized by ZFT. The measurement of chlorophyll fluorescence of Photosystem II [9] permits effective differentiation between living and merely fresh plants. Then ZFT selects only living plants for further processing.
To preserve the integrity of beneficial natural complexes and compounds, an ideal starting material would be a living plant lacking a mechanical "skeleton" of cell walls. As large indus-

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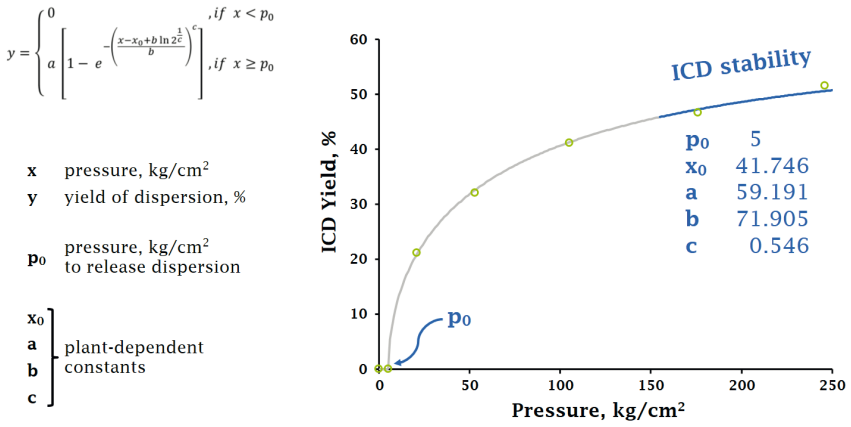

Fig. 1 Yield Curve Parameters for Intracellular Colloidal Dispersion from Living Sage (*Salvia officinalis*)

trial scale culturing of protoplasts is not feasible yet and does not reproduce the environment-driven metabolic conditions needed for the plant to express all natural complexes and compounds (including secondary metabolites), a practical alternative is the prompt separation of an intracellular colloidal dispersion from cell walls by mechanical forces.

Obtaining relatively stable intracellular colloidal dispersion (ICD) that exactly matches the original cell contents is achievable only with external enzymes because mechanical pressure does not move all types of living plant cell contents (e.g., organelles, natural complexes and compounds) with equal speed or ease. However, practical asymptotic approximation is possible without external chemicals (e.g. enzymes) by reaching a further critical area of stability (S) on the yield curve, such as the one demonstrated in **Fig. 1**. **Fig. 1** illustrates the curve parameters for living Sage.

Other plant species result in different plant-dependent parameters and conditions for reaching a close match to the original cell contents and level of stability. The Weibull distribution model [10] provided the best fit for these experimental data.

A separated relatively stable ICD can be immediately processed with a special ZFT Mobile Unit, which has the capability to process ~10 tons of living plants at a time. This strategy of bringing technology to the plants, rather than the reverse, prevents unwanted catabolic processes from occurring and improves both the preservation of natural complexes and compounds and the level of reproducibility. Physico-chemical testing of ICDs obtained from representative plants of 14 families showed factors with both high variability (e.g., ~80 % for solids content, ~50 % for conductivity, ~40 % for pH) and very low variability (e.g., ~5 % for osmolality, ~5 % for dielectric constants). Thus, the plants' osmolality [11] and dielectric constants [12] were identified and utilized as key parameters leading to uniformity and applicability for all plant species. A remarkably low variability of dielectric constant (real component) is highlighted by **Fig. 2**, displaying the mean and 95 % confidence interval for ICDs obtained from various plants selected from 14 families.

ZFT considers the obtained intracellular material as a relatively stable colloidal dispersion comprised of a continuous phase (cytoplasm and vacuole contents) and a dispersed phase (suspended organelles and their fragments). According to Derjaguin-Laundau-Verwey-Overbeek (DLVO) theory, this stability is maintained by the sum of van der Waals attractive and electrical double layer repulsive forces [13]. The energy barrier resulting from the repulsive force prevents particles of the dispersed phase from approaching unless they have sufficient energy to overcome that barrier, in which case the attractive force will pull them into contact (where they will irreversibly adhere). DLVO theory describes the interaction and potential energy of the particles based on their parameters, their distance from each other and characteristics of the continuous phase. Altering values of the variables affecting the repulsive force affects the stability of the dispersion, as displayed in **Fig. 3**.

Fig. 3 shows the particles as stylized chloroplasts. The effect of the double-layer repulsive force (V_D) is shown as thin dot-

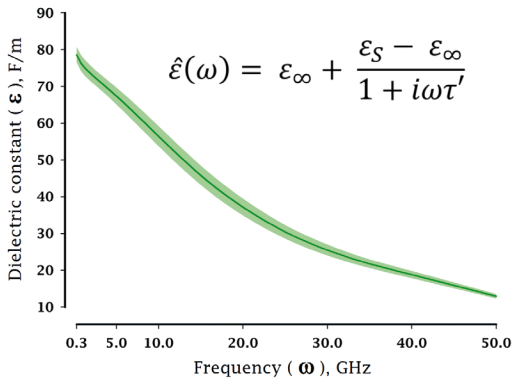

Fig. 2 Debye Equation and Dielectric Constants (Real Component) of ICDs from Various Plants Selected from 14 families

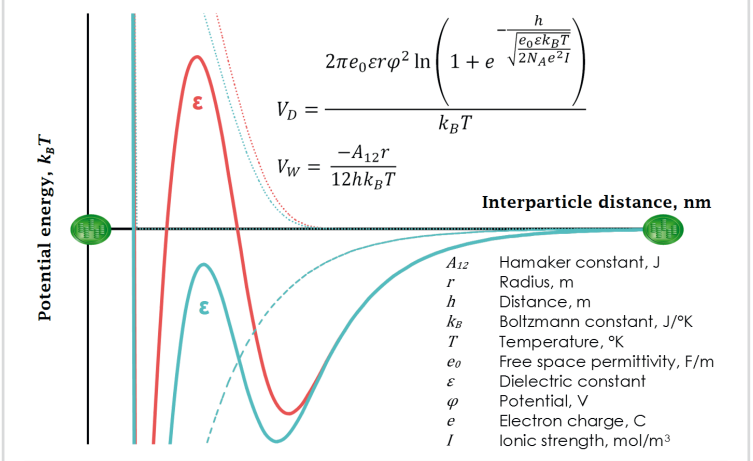

Fig. 3 Stability of Colloidal Dispersion and Effect of Dielectric Constant Change

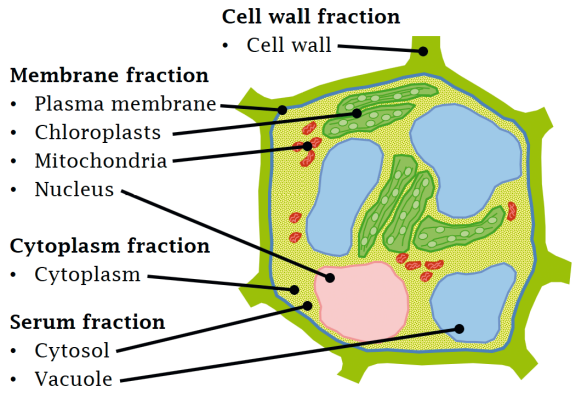

Fig. 4 Distribution of Living Plant Cell Components among Zeta Fractions

ted lines, while the effect of van der Waals attractive force (V_W) is shown as a thin dashed line. The effect of the sum of these forces with the steric repulsion force is shown as thick solid lines. Under normal conditions of colloidal stability (red color), an increase in potential energy as particles approach each other provides a potential energy barrier that is impossible to overcome without external energy input. This energy barrier keeps the particles separated, and the dispersion stable. Altered conditions (teal color) allow the double-layer repulsive force to decrease to the point where potential energy barrier does not exist, and particles may approach and agglomerate freely.

Restoration of the initial conditions does not then return stability, as particles have irreversibly agglomerated, and became easily removable by mechanical means.

One of the principles allowing such alteration of properties is the dependence of the dielectric constant (ε) on the frequency of the electromagnetic field (ω), as shown by Debye equation [12] in Fig. 2.

Utilizing DLVO theory and fundamental scientific principles discovered by Van't-Hoff and Debye, ZFT is based on targeted destabilization of intracellular colloidal dispersions, followed by their separation into various Zeta Fractions. The key steps in this process include collection of living plants with maximum metabolic activity; separation of the relatively stable ICD from the cell walls; treatment of the ICD to engage particular components of the dispersed phase and continuous phase in specific interactions by changing the balance between repulsive and attractive forces; and separation of ICD into different Zeta Fractions as displayed in Fig. 4.

It was found that the specific energy required to achieve targeted destabilization of ICD is much lower than the energy of chemical bonds, therefore preventing changes in molecular structure of components during the ZFT process [8]. Special continuous flow equipment was designed, built and is presently being explored for its capacity to accommodate large scale production of Zeta Fractions.

Sustainability

As an example of the benefits of such solvent-free technology, an evaluation of the environmental and sustainability aspects of ZFT was conducted as a cradle-to-grave analysis according to ISO 14040 and ISO 14044 standards using the ecoinvent database with the GaBi life-cycle assessment software package (PE International). The reference solvent system utilized for the comparative assessment included components with a dielectric constant from 2.0 to 80.0 F/m. A comparison of the environmental impact of producing 1000 kg of ingredient from a living Tea plant using the ZFT versus conventional solvent extraction is presented in Fig. 5.

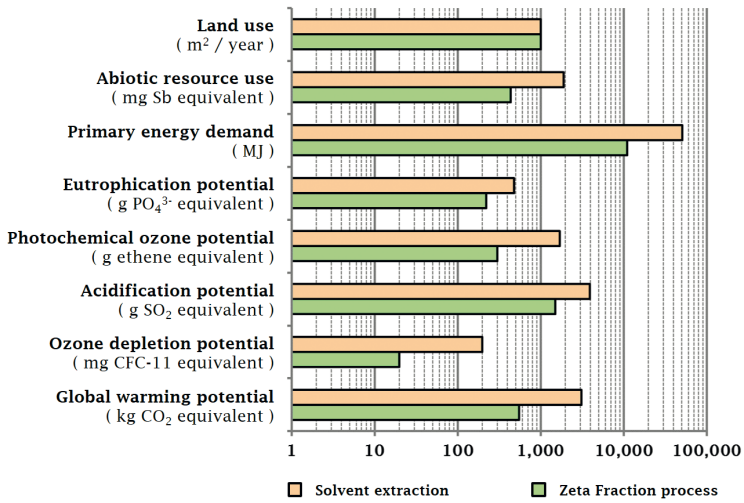

Fig. 5 The Environmental Impact of Producing Ingredient from Living Tea plant (*Camellia sinensis*) using the ZFT process versus conventional solvent extraction

This assessment of Zeta Fraction technology superiority would be even more favorable if the assessment had considered the safety risks of exposing workers and environment to hazardous solvents.

The composition of fractions obtained by ZFT notably differs from corresponding extracts obtained by conventional processes, as it shows both a greater abundance and a different distribution of desirable compounds. As an example, *Camellia sinensis* serum fraction, cytoplasm fraction, and both traditional green and black tea infusions were obtained in parallel from the identical cultivar. The corresponding analytical data related to catechins is presented in **Fig. 6**.

Safety and Reproducibility

While the diversity of natural compounds and complexes contained within plants provides many opportunities for finding potent properties and helpful synergies in reaching functionality, it also makes achieving safety a challenging proposition.

ZFT has been successfully used to remove specific undesirable components and chemicals of concern from biofunctional ingredients obtained from different plants. Examples of removed substances include parthenolide [6], a known skin sensitizer that can induce contact dermatitis (**Fig. 7**); proteins and high molecular weight peptides (respiratory sensitizers); patulin (mycotoxin associated with dermal toxicity); pheophorbides (photosensitizers, psoralens (photosensitizers); and heavy metals. In addition, the microbiological profiles for all research and commercial ingredients prepared via ZFT were consistently better than industry standards. Data presented in **Fig. 7** demonstrates an example of the remarkable reproducibility of 20 commercial batches (~ 500 kg each) of Zeta Fraction from Feverfew obtained in several consecutive years from different growers. It is particularly notable that these batches were obtained in several consecutive years and with source materials obtained from different growers. The yield per acre can vary from season to season but not the compositions of Zeta Fractions, which are genetically pre-determined.

Mutifunctionality

Multifunctionality is a highly desirable quality because healthy and attractive skin appearance is a result of complex processes and pathways. Skin irritation, inflammation, and hyperpigmentation are common and complex problems of great cosmetic importance. Addressing them requires a coordinated effect on multiple pathways, which is not always achievable by solitary compounds. The novel ingredient obtained from living *Nelumbo nucifera* (Sacred Lotus) by ZFT is a prime example of one that demonstrated desirable multifunctional properties (as reflected below in **Tab. 1**).

In addition, Lotus Zeta Fraction was further evaluated at Ashland Global Skin Research Center (Sofia Antipolis, France). It was observed that this ingredient was associated with: (1) increases in hyaluronic acid, filaggrin, and AQP3 expressions and a decrease in melanin expression in *ex vivo* human skin; (2) an increase in collagen 1 expression in adult human fibroblasts; (3) improved organization of elastic fibers when the skin was pretreated with the ingredient in *ex vivo* human skin; and (4) a reduction in SDS stress-induced barrier disruption in 3D reconstituted human epidermis. In clinical studies, the ingredients were also observed to (1) provide an improvement in skin moisturization and a reduction of transepidermal water loss, while promoting the improvement of skin softness,

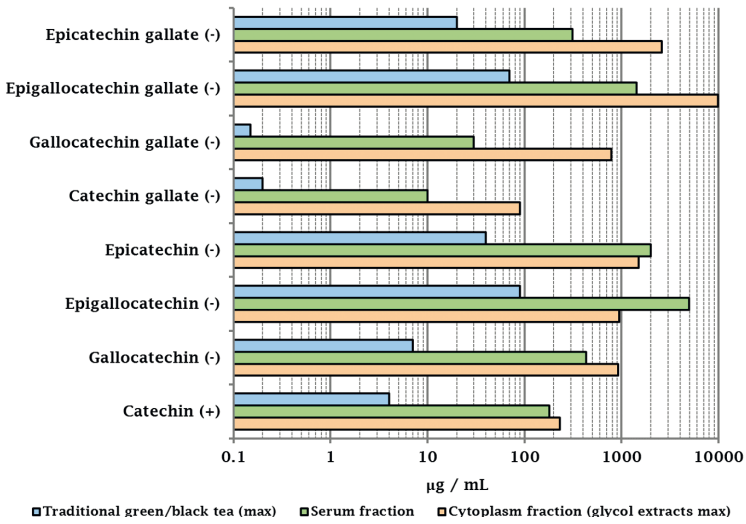

Fig. 6 Compositions of Zeta Fractions from *Camellia sinensis* versus Traditional Infusions

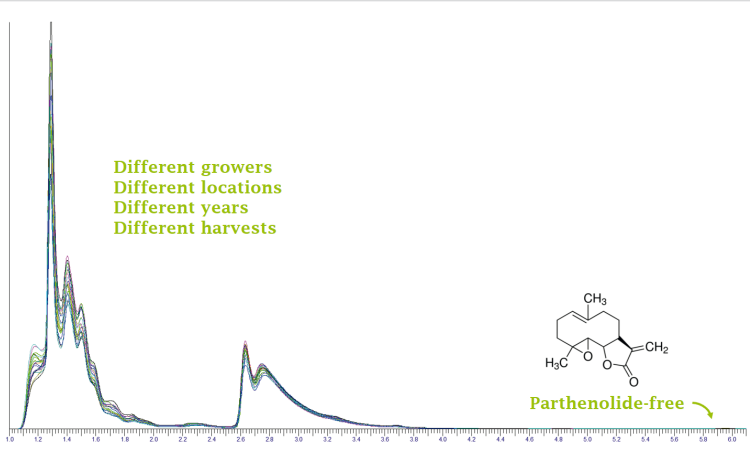

Fig. 7 Zeta Fraction from Feverfew is Free from Chemicals of Concern (parthenolide)

	Assay	Result (for "as is" fraction containing 6.60 % dry matter)
Oxidation (*in vitro*)	ORAC (antioxidant)	1 g = 31 mg (R)-Trolox methyl ether
	DPPH (quenching)	1 g quenches 26 mg DPPH radicals
Inflammation (Human Epidermal Keratinocyte model)	HEK Sun-induced Prostaglandin E_2	$IC_{50} \leq 0.05$ %
	HEK Sun-induced Interleukin 8	$IC_{50} \leq 0.005$ %
	HEK Sun-induced Interleukin 8	$IC_{50} = 0.02$ %
Proteases (Enzyme inhibition)	Elastase	$IC_{50} = 0.33$ %
	Matrix Metallopeptidase 3	$IC_{50} \leq 0.03$ %
Pigmentation (B16 melanocyte model)	Tyrosinase activity	$IC_{50} \approx 0.5$ %
	Tyrosinase Related Protein 1 activity	$IC_{50} \approx 0.75$ %
	Tyrosinase Related Protein 2 activity	$IC_{50} \approx 0.6$ %
	Melanin production	$IC_{50} \approx 0.77$ % (0.2 % decreases melanin by 25 %)

Tab. 1 *In vitro* Multifunctional Properties of Zeta Fraction from *Nelumbo nucifera*

compared to the placebo sides; (2) improve the appearance of wrinkles (number, volume, area) and skin roughness; and (3) improve skin appearance aspects such as drainage and body contour.

The safety and toxicological profile of this ingredient obtained from ZFT indicated that it is: a non-skin and eye irritant (Reconstructed Human Epidermis, 48-Hr Human Patch, Hen's Egg Chorioallantoic Membrane, Reconstituted Human Corneal Epithelium at 100 %), non-sensitizer (Human Repeat Insult Patch at 10 %, N > 200), non-phototoxic (3T3 Neutral Red Uptake Phototoxicity), and non-genotoxic ("Ames" bacterial reverse mutation).

Conclusion

Proprietary ZFT allows for the isolation of multifunctional natural complexes and compounds from living plants. This technology permits isolation of constituent parts of cells without the use of external solvents or even water, and with minimum energy consumption. ZFT creates minimal negative environmental impact and waste. It assures full control of the supply chain from cultivation through production, and enables new supporting intellectual property for resulting Zeta Fractions. Fractions produced by ZFT demonstrate superior efficacy, safety and reproducibility, and are able to target multiple pathways with a single ingredient. ZFT utilizes the underexplored potential of living plants and may be used in combination with existing extraction technologies to achieve effective volume reduction.

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